

Protein**Reviews**

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Volume 4:

Protein Misfolding, Aggregation, and Conformational Diseases

Part A: Protein Aggregation and Conformational Diseases

Edited by **Vladimir N. Uversky** and
Anthony L. Fink

Protein Misfolding, Aggregation, and Conformational Diseases

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Protein Misfolding, Aggregation, and Conformational Diseases

Part A: Protein Aggregation and Conformational Diseases

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Structural and Conformational Prerequisites of Amyloidogenesis

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1. Abstract

Recent reports give strong support to the idea that amyloid fibril formation and the subsequent development of protein deposition diseases originate from conformational changes in corresponding amyloidogenic proteins. In this review recent findings are surveyed to illustrate that protein fibrillogenesis requires a partially folded conformation. This amyloidogenic conformation is relatively unfolded, and shares many structural properties with the premolten globule state, a partially folded intermediate frequently observed in the early stages of protein folding and under some equilibrium conditions. The inherent flexibility of such an intermediate is essential in allowing the conformational rearrangements necessary to form the core cross- β structure of the amyloid fibril.

2. Introduction

A significant number of human diseases, including many neurodegenerative disorders and the amyloidoses, originate from the deposition of stable, ordered, filamentous protein aggregates, known as amyloid fibrils. In each of these pathological states, a specific protein or protein fragment changes from its natural soluble form into insoluble fibrils, which accumulate in a variety of organs and tissues (Kelly, 1998; Dobson, 1999; Bellotti *et al.*, 1999; Uversky *et al.*, 1999b, 1999c; Rochet and Lansbury, 2000; Uversky and Fink, 2004). Although approximately 20 different proteins are known to be involved in the amyloidoses (extracellular deposits), they are mostly unrelated in terms of sequence or structure. In addition, a number of diseases also involve the deposition of fibrillar intracellular deposits, as well as nonfibrillar deposits.

Prior to fibrillation, amyloidogenic polypeptides may be rich in β -sheet, α -helix, β -helix, or contain both α -helices and β -sheets (see Table 1-1). They may be globular proteins with rigid 3D-structure or belong to the class of natively unfolded (or intrinsically unstructured) proteins. Despite these differences, the fibrils from different pathologies display many common properties including a core cross- β -sheet structure in which continuous β -sheets are formed with β -strands running perpendicular to the long axis of the fibrils (Sunde *et al.*, 1997). All fibrils have similar morphologies, being twisted, rope-like structures, reflecting a filamentous substructure. Amyloid fibrils have been formed *in vitro* from disease-associated as well as from disease-unrelated proteins and peptides (see Table 1-1). Moreover, there is an increasing belief that the ability to fibrillate is a generic property of a polypeptide chain, and all proteins are potentially able to form amyloid fibrils under appropriate conditions (Dobson, 1999, 2001; Fandrich *et al.*, 2001; Pertinhez *et al.*, 2001).

Table 1-1. Amyloidogenic proteins and amyloid-based clinical disorders

Disease-associated amyloidogenic proteins			
Amyloidogenic protein	Type of structure	Disease	Tissue distribution of protein deposits
Prion protein and its fragments	N-terminal fragment (23–121) is natively unfolded; C-terminal domain (121–230) is α -helical (predominantly)	Creutzfeld-Jacob disease (CJD) Gerstmann-Straussler-Schneiker syndrome (GSS) Fatal familial insomnia (FFI) Kuru Bovine spongiform encephalopathy (BSE) and scrapie	Brain
Cystatin C	α/β	Hereditary cystatin c amyloid angiopathy (HCCAA)	Brain
Amyloid- β and its fragments	Natively unfolded	Alzheimer disease (AD) Dutch hereditary cerebral hemorrhage with amyloidosis (HCHWA, also known as cerebrovascular amyloidosis) Congophilic angiopathy	Brain
Abri	Natively unfolded	Familial British dementia	Brain, spinal cord
Huntingtin	α -Helical (but exon 1 is unfolded and forms fibrils)	Huntington Disease	Brain
Androgen receptor protein	Ligand-binding (LBD) and DNA-binding domains (DBD) are α -helical; amino-terminal domain (NTD) is natively unfolded	Spinal and bulbar muscular atrophy (SBMA)	Brain, scrotal skin, dermis, kidney, heart, and testis, spinal cord
Ataxin-1	Unknown (likely natively unfolded)	Spinocerebellar ataxia (SCA) Neuronal intranuclear inclusion disease (NIID)	Brain, spinal cord Central and peripheral nervous system
DRPLA protein (atrophin-1)	Unknown (likely natively unfolded)	Hereditary dentatorubral-pallidoluysian atrophy (DRPLA)	Brain
Serum amyloid A and its fragments	α/β	AA amyloidosis (inflammation-associated reactive systemic amyloidosis)	Bladder, stomach, thyroid, kidney, liver, spleen, gastrointestinal tract
Medin (245–294 fragment of lactadherin)	β -Sheet	Aortic medial amyloidosis	Aortic smooth muscle
Islet amyloid polypeptide (IAPP, Amylin)	Natively unfolded	Pancreatic islet amyloidosis in late-onset diabetes (type II diabetes mellitus)	Pancreas
Calcitonin	Natively unfolded	Medullary carcinoma of the thyroid (MCT)	Thyroid
Lysozyme	$\alpha + \beta$	Hereditary systemic amyloidosis	Several visceral organs and tissues

Table 1-1. Continued

Disease-associated amyloidogenic proteins			
Amyloidogenic protein	Type of structure	Disease	Tissue distribution of protein deposits
Gelsolin	α/β	Hereditary systemic amyloidosis Finnish-type familial amyloidosis	Several visceral organs and tissues
Transthyretin	β -Sheet (predominantly)	Senile systemic amyloidosis (SSA) (or senile cardiac amyloidosis)	Almost all organs and tissues, including heart, gland, arteries, bones, liver, digestive tract, etc. Various organs and tissues.
Apolipoprotein A1	α -Helical	Familial amyloid polyneuropathy (FAP) Hereditary systemic amyloidosis	Eyes
β_2 -Microglobulin	β -Sheet	Amyloid associated with hemodialysis (AH or A β 2M) (athropathy in hemodialysis)	Musculoskeletal tissues (large and medium-sized joints, bones, muscles), peripheral nervous system, gastrointestinal tract, tongue, heart, urogenital tract
Tau protein	Probably natively unfolded	Alzheimer disease (AD), Pick's disease, Progressive supranuclear palsy (PSP)	Brain
Fibrinogen and its fragments	β -Sheet	Hereditary renal amyloidosis	Kidney
Immunoglobulin light chain variable domains	β -Sheet	Light chain associated amyloidosis or AL amyloidosis	Almost all organs and tissues, including heart, kidneys, liver, spleen, gastrointestinal tract, skin, tongue, endocrine glands, peripheral nervous system, etc. Liver, spleen, bone marrow, vessel walls, parenchymal tissue, kidneys, heart, liver, skin, lungs, tongue, ovary, pancreas, etc. Heart
Atrial natriuretic factor	"Small protein"	Light chain deposition disease or LCDD Light chain cast nephropathy Light chain cardiomyopathy Atrial amyloidosis	Heart
Insulin	Predominantly α -helical	Injection-localized amyloidosis	Skin, muscles

(Continued)

Table 1-1. Amyloidogenic proteins and amyloid-based clinical disorders (*Continued*)

Disease-associated amyloidogenic proteins			
Amyloidogenic protein	Type of structure	Disease	Tissue distribution of protein deposits
α -Synuclein	Natively unfolded	Parkinson's disease (PD) Diffuse Lewy bodies disease (DLBD) Lewy bodies variant of Alzheimer's disease (LBVAD) Dementia with Lewy bodies (DLB) Multiple system atrophy (MSA) Hallervorden-Spatz disease	Brain
NAC (central fragment of α -synuclein)	Natively unfolded	Alzheimer disease (AD)	Brain
Nondisease-related amyloidogenic proteins and peptides			
Protein (peptide), reference	Type of structure	Protein (peptide), reference	Type of structure
Betabellins 15D and 16D	β -Sandwich	Prothymosin α	Natively unfolded
Cytochrome c_{552}	α -Helical	Myoglobin	α -Helical
Methionine aminopeptidase	α -Helical	Muscle acylphosphatase	α/β
Phosphoglycerate kinase	α/β	Hen egg white lysozyme	$\alpha + \beta$
Hen egg white lysozyme, β -domain	β -Sheet	Acidic fibroblast growth factor	β -Barrel
PI3-SH3 domain	β -Barrel	OspA protein, BH ⁹⁻¹⁰ peptide	β -Turn
β -Lactoglobulin	β -Sheet (predominantly)	<i>De novo</i> $\alpha\alpha$ peptide	α -Helix-turn- α -helix
Monellin	α/β	Lung surfactant protein C	α -Helix
Immunoglobulin light chain LEN	β -Sheet	α -Lactalbumin	$\alpha + \beta$
HypF, N-terminal domain,	α/β	V _L domain of mouse antibody F11	β -Sheet
Human complement receptor 1, 18–34 fragment	Unfolded	Apolipoprotein C-II	Natively unfolded
Human stefin B	α/β	Cold shock protein A	β -Barrel
GAGA factor	Natively unfolded	Protein G, B1 Ig-binding domain	Four-stranded β -sheet with a flanking α -helix
Yeast prion Ure2p	α -Helical/unfolded	Cold shock protein B, 1–22 fragment	Unfolded
Herpes simplex virus glycoprotein B fragment	β -Structural	<i>De novo</i> proteins from combinatorial library	β -Structural
The fiber protein of adenovirus, 355–396 peptide from shaft	Fibrillar	Soluble homopolypeptides,: poly-L-lysine poly-L-glutamic acid poly-L-threonine	Unordered

In other words, accumulated evidences suggest that amyloidogenic aggregation, being often a pathogenic state of proteins, is a generic phase of any peptide chain, a way of organizing in three dimensions dominated by main-chain interactions and essentially oblivious of the information encoded in the primary sequence. On the other hand, natural soluble proteins are known to spontaneously fold into single-molecule conformations of marginal stability, often requiring binding partnerships or complexation to preserve the integrity of the so-called native fold (Fernandez and Scheraga, 2003).

Although the folding process and its final stable outcome are very much dependent on the amino acid composition of the chain (Anfinsen, 1973), the amyloid state appears to be fairly insensitive to the information encoded in the side chains: at first sight, amyloidogenic aggregation does not seem to require an "aggregation code." But on second thoughts, it must place severe constraints on the primary sequence, as some proteins tend to be relatively prone to aggregate even under physiological conditions (Fernandez *et al.*, 2003), while others require extreme conditions to do so (Dobson, 1999, 2001, 2002; Uversky and Fink, 2004). In addition, negative-design features of the folded state have been recognized as responsible for averting aggregation (Richardson and Richardson, 2002). Thus, it is not entirely correct to characterize the aberrant aggregation as a "polymer physics phase," shared by polypeptides with arbitrary, suboptimal, or random sequence, in contrast with the folded state, whose existence and integrity is determined typically unambiguously by the primary sequence.

Clearly, a selection pressure must operate to optimize the primary sequence, so it can render a stable soluble structure. This optimization is needed to prevent the functionally competent fold from reverting to the primeval amyloid phase. On the other hand, certain sequences are better optimized to escape aggregation than others even under conditions known to sustain the native fold (Fernandez *et al.*, 2003). What generic structural feature of the native fold confers a protection from aggregation?

Although amyloidogenic aggregation has been shown to be always plausible provided sufficiently stringent denaturing conditions are applied (Dobson, 1999, 2001, 2002; Uversky and Fink, 2004), a marked amyloidogenic propensity has been detected on a number of proteins under physiological or near-physiological conditions, particularly if the monomeric folding domain is deprived of its natural interacting partners (Fernandez and Berry, 2003). Such findings suggest not only that not all soluble structures have been optimized to the same degree to avert aggregation, but also, that the more reliant the structure is on binding partnerships or complexation, the more vulnerable it becomes in regard to reverting to the primeval phase. Thus, an overexpression of a folding domain with high complexation requirements *in vivo*, or the modification of its binding partners as a result of genetic accidents, or any factor that distorts its natural interactive context are likely to bolster a transition to an amyloidogenic state (Fernandez *et al.*, 2003). These observations prompt us to pose the following question: *What type of deficiency in the native fold constitutes a signal for aberrant aggregation?*

As far as the majority of proteins is concerned (i.e., those that do not easily fibrillate under physiological conditions), it has been already pointed out that their aggregation requires an application of some denaturing conditions (Dobson, 1999, 2001, 2002; Uversky and Fink, 2004). In other words, it has been proposed that fibrillation of these proteins can occur when their rigid native structure is *destabilized*, favoring formation of a *partially unfolded conformation* (Fink, 1998; Kelly, 1998; Bellotti *et al.*, 1999; Dobson, 1999; Lansbury, 1999; Uversky *et al.*, 1999b, 1999c; Rochet and Lansbury, 2000; Canet *et al.*, 2001; Zerovnik, 2002; Uversky and Fink, 2004). This model, however, cannot be directly applied to intrinsically unstructured (natively unfolded) proteins, as they are devoid of secondary structure to start with. Instead, the primary step of their fibrillogenesis might involve *stabilization of a partially folded conformation*; that is, partial folding rather than unfolding has to occur (Schweers *et al.*, 1994; Teplov, 1998; Kaye *et al.*, 1999; Uversky *et al.*, 2001a; Goers *et al.*, 2002; Uversky and Fink, 2004). Thus, by taking the intrinsically unstructured proteins into consid-

eration, a general hypothesis of fibrillogenesis might be formulated as follows: *structural transformation of a polypeptide chain into a partially folded conformation represents an important prerequisite for protein fibrillation*. Presumably, such partially folded conformation enables specific intermolecular interactions, including electrostatic attraction, hydrogen bonding, and hydrophobic contacts, which are necessary for oligomerization and fibrillation. All this raises another important question: *What are the conformational prerequisites for partially folded intermediates to become the amyloidogenic species?* We shall try to answer these questions below.

3. What Kind of Defects in the Soluble Folded State Bolster the Conversion to the Amyloidogenic Phase?

A recent assessment of the so-called *wrapping* of a soluble folded structure (Fernandez, 2001, 2002, 2004; Fernandez *et al.*, 2002, 2003; Fernandez and Scott, 2003a, 2003b, 2003c) might prove paramount to come to grips with this problem. In the native structures reported in the protein database (PDB), most, *but not all*, backbone hydrogen bonds are completely wrapped intramolecularly by surrounding the electrostatically interactive amide-carbonyl pair with nonpolar groups from side chains. On the other hand, the underwrapped (insufficiently desolvated) hydrogen bonds have been shown to be markers for protein interactivity (Fernandez and Scheraga, 2003) and typically conserved as a domain fold is examined across species (Fernandez, 2004). Furthermore, at concentrations higher than four wrapping defects per 1000 Å² on the protein surface, wrapping deficiencies become indicative of protein aggregation (Fernandez *et al.*, 2003). This observation turns the wrapping analysis into a powerful diagnosis tool. The only precursor to this line of thinking appears to be the observation that backbone solvation could be regarded as an important factor in determining the β propensity of amino acids (Avbelj and Baldwin, 2002).

The implied stickiness of such wrapping defects has been corroborated experimentally (Fernandez and Scott, 2003a), calculated theoretically in solid agreement (Fernandez and Scott, 2003c), and statistically inferred from proteomic data (Fernandez and Scheraga, 2003). This stickiness arises because the exogenous removal of surrounding water, which results upon protein associations contributes to descreen the partial charges on the backbone amide and carbonyl and thus enhances the hydrogen-bond electrostatics, stabilizing the bond. This stabilization may be rationalized as resulting from the destabilization of the nonbonded state: the exposed (nonbonding) amide and carbonyl would be hindered from being solvated as a nonpolar group from a binding partner enters the dehydration domain of the pre-formed underwrapped hydrogen bond.

The condition of “keeping the structure dry in water” becomes a requirement to preserve the structural integrity of soluble proteins and imposes a severe building constraint (and thereby an evolutionary pressure) on such proteins. Thus, it is expected that the optimization of the structures resulting from this type of evolutionary constraint would be uneven over a range of soluble proteins, resulting in marked differences in aggregation propensity.

All in all, this analysis of the backbone desolvation of the native state supports and clarifies the physical picture put forth by Dobson (Dobson, 1999, 2001), in which amyloidogenic propensity depends crucially on the fact that main-chain interactions become dominant in detriment of the amino acid sequence that encodes the folded state. Precisely, main-chain interactions may dominate as the main chain of the folded state is not properly protected from water attack (Fernandez *et al.*, 2003). It is instructive to compare this statement with the local analysis of Avbelj and Baldwin (2002) in the sense that backbone solvation is a determinant of β propensity. Thus, an overexposed backbone hydrogen bond in the native fold is an indicator of a failure in folding cooperativity, as it reveals an inability to remove water from an interactive polar pair by means of a many-body correlation, and at the same time, it is a signal enabling the diagnosis of amyloidogenic propensity.

Often, the inability to properly wrap a structure intramolecularly is compensated by protein complexation. This clarifies the physical picture, suggesting that the more dependent the folding domain is on its interactive partnerships to preserve its structure, the more likely it is to be prone to revert to its primeval aggregated phase. In essence, we could regard these competing structural alternatives as reflecting a struggle for the survival of backbone hydrogen bonds.

The assessment of packing defects enables us to discern that some soluble proteins may have been better optimized to avoid amyloidogenic aggregation than others. For instance, it is revealing to contrast the pattern of underwrapped sites in human hemoglobin β -subunit (pdb.1bz0), a good backbone wrapper, against the ordered region of the human cellular prion protein (pdb.1qm0) (Prusiner, 1998; Fernandez, 2002). Although about 3% of the backbone hydrogen bonds in hemoglobin are underwrapped, nearly 50% of those of the cellular prion protein are underwrapped. Direct inspection of the pattern of desolvation of the main chain clearly reveals that the prion cellular fold is too vulnerable to water attack and at the same time too sticky to avert aggregation. Clearly, its sequence has not been optimized to “keep the backbone hydrogen bonds dry” in the folded state. In fact, *their extent of exposure of backbone hydrogen bonds is the highest among soluble proteins in the entire PDB*, with the sole exception of some toxins whose stable fold is held together by a profusion of disulfide bridges (Fernandez and Scheraga, 2003).

It is suggestive that an inability to protect the main chain is precisely the type of deficiency that best correlates with a propensity to reverse to a primeval aggregation phase determined by main-chain interactions. The actual mechanism by which such defects induce or nucleate the transition is still opaque, although the inherent adhesiveness of packing defects obviously plays a role.

To conclude, we may include some evolutionary remarks. A paradigmatic discovery in biology revealed that folds are conserved across species to perform specific functions. However, the wrapping of such folds is clearly not conserved (Fernandez *et al.*, 2004). Moreover, the wrapping of a conserved fold in species of complex physiology tends to be far worse than in primitive organisms. As a rule, any fold in *Archea* or *Prokarya* would tend to be far better wrapped than the same fold in *Eukarya* (Fernandez *et al.*, 2004). This fact suggests how complex physiologies may be achieved without dramatically expanding genome size, a standing problem in biology. Considerable network complexity may be achieved by actually fostering a higher level of complexation or binding partnership, as dictated by an increasingly more precarious wrapping of the isolated folding domains. Of course, according to our previous analysis, such complex design entails an inherent danger: the reversal of highly underwrapped folding domains to an amyloidogenic phase even under physiological conditions. It would be interesting to assess the extent to which amyloidosis-related aberrant states may arise in *Archea* under conditions that would sustain the native folds. We would predict such states to be rare. On the other hand, amyloidosis is likely to be a consequence of high complexity in proteomic connectivity, as dictated by the structural fragility of highly interactive proteins. Thus, the relationship between network centrality, structural wrapping, and propensity for aberrant aggregation will be investigated in forthcoming work.

4. What Are the Conformational Prerequisites for Partially Folded Intermediates to Become the Amyloidogenic Species?

4.1. The Requirement for Partial Unfolding: Fibrillogenesis of Globular Proteins

Data are accumulated supporting the model where the first critical step in fibrillogenesis of globular proteins is their partial unfolding (Fink, 1998; Kelly, 1998; Bellotti *et al.*, 1999; Dobson, 1999, 2001; Lansbury, 1999; Uversky *et al.*, 1999b, 1999c; Rochet and Lansbury, 2000; Zerovnik,

2002). Due to conformational breathing, the structure of a globular protein under physiological conditions represents a mixture of tightly folded and multiple partially unfolded conformations, with the former greatly predominating (Englander and Kallenbach, 1983; Chamberlain *et al.*, 1996). Most mutations associated with accelerated fibrillation and protein deposition diseases have been shown to destabilize the native structure, increasing the steady-state concentration of partially folded conformers (Kelly, 1996, 1998; Wetzel, 1997; Canet *et al.*, 1999; Dobson, 1999; Lashuel *et al.*, 1999; Rochet and Lansbury, 2000; Heegaard *et al.*, 2001; Nielsen *et al.*, 2001a; Saraiva, 2001). Conversely, the amyloidogenicity of a protein can be significantly reduced by stabilization of the native structure (Nielsen *et al.*, 2001b), for example, via specific binding of ligands (Baures *et al.*, 1998; Peterson *et al.*, 1998; Oza *et al.*, 1999, 2002; Klabunde *et al.*, 2000; Chiti *et al.*, 2001; Nielsen *et al.*, 2001b; McCammon *et al.*, 2002; Raghu *et al.*, 2002). Furthermore, it has been shown that destabilization of the native globular structure (by application of low or high pH, high temperatures, low to moderate concentrations of strong denaturants, organic solvents, etc.) may significantly accelerate the rate of fibril formation. Thus, amyloid fibril formation is promoted when significant accumulation of a relatively unfolded protein occurs; that is, via the native state destabilization under the conditions in which noncovalent interactions still remain favorable (Guijarro *et al.*, 1998; Litvinovich *et al.*, 1998; Chiti *et al.*, 1999; Konno *et al.*, 1999; Krebs *et al.*, 2000; Ramirez-Alvarado *et al.*, 2000; Yutani *et al.*, 2000; Hamada and Dobson, 2002). A few illustrative examples of proteins for which there is evidence for the formation of relatively unfolded amyloidogenic intermediates as critical species in fibrillation are considered below.

4.1.1. Transthyretin

Transthyretin (TTR), or prealbumin, is a β -structural homotetramer, which is found in human plasma (0.1–0.4 mg/ml) and cerebral spinal fluid (0.017 mg/ml), with the plasma form being the amyloidogenic precursor. Wild-type TTR amyloidogenesis may cause senile systemic amyloidosis, characterized by deposition and pathology in the heart after age 60 (Westermarck *et al.*, 1990). One of more than 80 single-site TTR variants causes early onset amyloid formation (as early as the second decade). These pathological conditions, being characterized by neuropathy and/or organ dysfunction, are responsible for a number of diseases collectively termed familial amyloid polyneuropathy (FAP; see Table 1-1) (Saraiva *et al.*, 1984). TTR can be converted into amyloid *in vitro* by dissociation of the tetramer into monomers with depleted tertiary structure (Lai *et al.*, 1996). Furthermore, a monomeric TTR variant with native-like structure and stability has been recently designed, which was shown to be nonamyloidogenic, unless partially unfolded (Jiang *et al.*, 2001). Conversely, ligands that bind to the native tetramer and stabilize it have been shown to minimize fibril formation (Baures *et al.*, 1998; Peterson *et al.*, 1998; Oza *et al.*, 1999, 2002; Klabunde *et al.*, 2000; Chiti *et al.*, 2001; McCammon *et al.*, 2002; Raghu *et al.*, 2002; Hammarstrom *et al.*, 2003).

4.1.2. β_2 -Microglobulin

β_2 -Microglobulin is a small protein with an immunoglobulin fold characterized by two anti-parallel pleated sheets of β -strands linked together by a disulfide bridge (Becker and Reeke, 1985). This protein is a normal constituent of plasma, where its concentration in adults is 1.1–2.7 mg/liter. The daily production of β_2 -microglobulin is in the range of 150–200 mg, of which 97% is excreted in the kidneys. However, blood dialysis therapy of patients with renal failure results in the retention of a high concentration of β_2 -microglobulin in the plasma. This leads to aggregation and fibrillation of this protein in joints and tissues, which is a hallmark of dialysis-related amyloidosis, a pathological condition affecting ~20% of patients with kidney failure within 2 years after the onset of hemodialysis

treatment. This disorder does not require a mutated, processed, or chemically modified precursor protein (Jadoul *et al.*, 1997). Fibrillogenesis of β_2 -microglobulin *in vitro* was shown to be accelerated by low pH and high ionic strength; that is, under conditions leading to the accumulation of a partially folded conformation with denatured tertiary structure, some secondary structure, high affinity to ANS, line broadening in one-dimensional ^1H -NMR spectra, and weakly protected from hydrogen exchange (McParland *et al.*, 2000, 2002; Hong *et al.*, 2002; Hoshino *et al.*, 2002). Overall, it has been concluded that although destabilization of the native state is important in the generation of amyloid fibrils, population of specific denatured states is a prerequisite for amyloid formation from β_2 -microglobulin.

4.1.3. Serum Amyloid A Protein

Serum amyloid A (SAA) is a family of apolipoprotein isoforms associated with high-density lipoprotein (Marhaug and Dowton, 1994). The serum concentration of SAA can increase from the healthy reference range of under 10mg/l to more than 1000mg/l during active inflammation, and these levels can persist indefinitely (Wilkins *et al.*, 1994). In some chronic inflammatory diseases, the amyloid fibrils may be derived from the acute-phase SAA protein, causing the reactive systemic (AA, secondary) amyloidosis. Structure and propensity to aggregate have been compared for several SAA isoforms. It has been shown that mouse SAA2 has about one-half of the α -helix content of the SAA1 analogue. Importantly, decreased helical content correlated with increased propensity to aggregate for the SAA2 form compared with SAA1 (McCubbin *et al.*, 1988).

4.1.4. Immunoglobulin Light Chains

A pathological condition known as light chain, or AL, amyloidosis affecting different organs, especially kidneys and heart, is caused by the systemic extracellular deposition of monoclonal immunoglobulin light chain variable domains in the form of insoluble amyloid fibrils (Buxbaum, 1992) (see Table 1-1). The accumulation of two partially folded intermediates has been associated with the exposure of one of the amyloidogenic light chain variable domains, SMA, to acidic pH (Khurana *et al.*, 2001). A relatively native-like intermediate, I_N , was observed between pH 4 and 6, which preferentially leads to amorphous aggregates. At pH below 3, a relatively unfolded, but compact, intermediate, I_U , was observed, which readily forms amyloid fibrils (Khurana *et al.*, 2001). Comparable data have been recently reported for another light chain variable domain, LEN (Souillac *et al.*, 2002a, 2002b). Furthermore, a general correlation between reduced thermodynamic stability and increased amyloidogenicity has been reported for number of other immunoglobulin light chains (Hurle *et al.*, 1994; Raffin *et al.*, 1999; Wall *et al.*, 1999a, 1999b; Bellotti *et al.*, 2000; Kim *et al.*, 2000).

4.1.5. Insulin

Insulin is a small α -helical protein-hormone that is crucial for the control of glucose metabolism and in diabetes treatment. Amyloid deposits comprised of fibrillar insulin have been observed both in patients with diabetes (Westermarck and Wilander, 1983; Westermarck *et al.*, 1987) and in normal aging (Ehrlich and Ratner, 1961), as well as after continuous subcutaneous insulin infusion and after repeated insulin injections (Brange *et al.*, 1997). In solution, insulin exists as an equilibrium mixture of monomers, dimers, tetramers, hexamers, and possibly higher associated states, depending on concentration, pH, metal ions, ionic strength, and solvent composition (Bryant *et al.*, 1993; Brange *et al.*, 1997). In 20% acetic acid insulin is monomeric and has a native-like conformation (Weiss

et al., 1989; Nielsen *et al.*, 2001b). The fibrillation of insulin *in vitro* was shown to be accelerated in the presence of denaturants such as urea, whereas stabilizers, such as trimethylamine *N*-oxide (TMAO) and sucrose, decrease the fibrillation rate (Nielsen *et al.*, 2001b). Based on these observations it has been proposed that the early stages of insulin fibril formation involve the dissociation of native associated states (hexamer, tetramer, and dimer) to give native monomer, which is in equilibrium with the fibrillation-competent relatively unfolded intermediate (Nielsen *et al.*, 2001b).

4.1.6. Human Lysozyme

The enzyme lysozyme is involved in an amyloid-related human disorder (Pepys *et al.*, 1993) in which the disease is associated with single-point mutations in the lysozyme gene, and fibrils are deposited widely in tissues (Table 1-1) (Pepys *et al.*, 1993). Wild-type human lysozyme and its two amyloidogenic variants have been found to form a partially folded state at low pH (Morozova-Roche *et al.*, 2000). This state was characterized by extensive disruption of tertiary interactions and partial loss of secondary structure. The amyloidogenic mutant proteins were significantly less stable than the wild-type protein, leading to higher populations of the partially unfolded intermediate and thus greater propensity to form fibrils (Morozova-Roche *et al.*, 2000).

4.1.7. α -Lactalbumin

α -Lactalbumin (α -LA) is a small acidic protein with a Ca^{2+} -binding site and a similar 3D fold to lysozyme. α -LA is comprised of a large α -helix domain and a small β -sheet domain connected by a calcium-binding loop and four disulfide bridges (Permyakov and Berliner, 2000). Although fibrillation of α -LA is not attributed to any pathological conditions, it was shown to form amyloid fibrils *in vitro* at low pH. S-carboxymethyl- α -lactalbumin, a disordered form of the protein with three of the disulfide bridges reduced, was even more susceptible to fibrillation. S-carboxymethyl- α -lactalbumin exhibits the properties of a premolten globule, and its fibrillation is orders of magnitude faster than when starting with the molten globule conformation (Goers *et al.*, 2002). Other partially folded conformations induced in α -LA at neutral pH did not fibrillate, although some precipitated out of solution as amorphous aggregates. Based on these data it was concluded that the transformation from native state to a substantially unfolded conformation is required for successful fibril formation, whereas less unfolded species may form amorphous aggregates (Goers *et al.*, 2002).

4.1.8. Monellin

Monellin is a plant protein isolated from the fruit of the tropical plant *Dioscoreophyllum cumminsii*. It is a sweet-tasting protein composed of two subunits, one being 45 residues (A chain) and the other 50 residues (B chain). X-ray and NMR methods indicate the structure of monellin has a five-strand antiparallel β -sheet and a 15-residue α -helix (Tomic *et al.*, 1992) (Table 1-1). Monellin undergoes irreversible thermal unfolding at pH 2.5 and 85°C, leading to an unfolded-like conformation (Konno *et al.*, 1999). Incubation under these conditions for 3 hours, leads to amyloid-like fibrils of ~10nm width, which were shown to bind Congo red (Konno *et al.*, 1999).

4.2. The Requirement for Partial Folding: Fibrillogenesis of Natively Unfolded Proteins

Certain proteins seem to require a high degree of structural disorder in their native states to fulfill their function (Wright and Dyson, 1999; Dunker *et al.*, 2001, 2002; Dyson and Wright, 2002;

Iakoucheva *et al.*, 2002; Uversky, 2002a, 2002b). We now consider details of the fibrillogenesis of such intrinsically unstructured proteins, which represent a significant portion of known amyloidogenic proteins (Table 1-1). Obviously, these proteins are better suited for amyloidogenesis, as they lack significant conformational constraints, being substantially more conformationally motile, and thus being able to polymerize more readily than tightly packed globular proteins. Substantial evidence suggests that the earliest stage of fibrillation of these proteins is their partial folding. The following section considers a few illustrative examples of fibril formation of natively unfolded proteins.

4.2.1. Amyloid β -Protein

Alzheimer's disease (AD) is the most prevalent age-dependent dementia, which is characterized pathologically by the accumulation of extracellular amyloid deposits in the cerebral cortex and vasculature, containing amyloid β -protein (A β), and of intracellular neurofibrillary tangles from the protein tau. A β is a 40–42-residue peptide produced by endoproteolytic cleavage of the amyloid β -protein precursor (APP). A β appears to be unfolded at the beginning of the fibrillation under physiological conditions (see Table 1-1). NMR studies have shown that monomers of A β (1–40), or A β (1–42) possess no α -helical or β -sheet structure (Kirkitadze *et al.*, 2001), that is, they exist predominately as random extended chains. Partial folding to the premolten globule-like conformation has been detected at the earliest stages of A β (1–40) fibrillation.

4.2.2. Tau Protein

Tau, a microtubule assembly protein isolated from brain microtubules, represents a family of isoforms. Heterogeneity is explained in part by alternative mRNA splicing leading to the appearance of one, two, three, or four repeats in the C-terminal region (Himmler, 1989). Posttranslational phosphorylation of tau is an additional source of microheterogeneity (Kenessey and Yen, 1993). Aggregation of tau protein in neuronal cells is attributed to the progress of AD and various other neurodegenerative disorders, especially frontotemporal dementia (Crowther and Goedert, 2000; Goedert, 2001b). In these cases specific tau-containing neurofibrillary tangles (paired helical filaments, PHFs) are formed (Delacourte and Buee, 1997). Hyperphosphorylation was shown to be a common characteristic of pathological tau (Vulliet *et al.*, 1992), with 10 major phosphorylation sites being identified in tau isolated from PHFs (Morishimakawashima *et al.*, 1995). Hyperphosphorylation was shown to be accompanied by the transformation from the unfolded state of tau into a partially folded conformation (Hagstedt *et al.*, 1989; Uversky *et al.*, 1998), accelerating dramatically the self-assembly of this protein into PHFs *in vitro* (Alonso *et al.*, 1996).

4.2.3. α -Synuclein

α -Synuclein is a small (14 kDa), soluble, intracellular, highly conserved protein that is abundant in various regions of the brain. Structurally, purified α -synuclein is a typical natively unfolded protein (Weinreb *et al.*, 1996; Uversky *et al.*, 2001a) (Table 1-1). Several observations indicate that this pre-synaptic protein is involved in the pathogenesis of Parkinson's disease (PD) (Masliah *et al.*, 2000; Link, 2001; Giasson *et al.*, 2002; Lee *et al.*, 2002). Interestingly, the peptide derived from the central hydrophobic region of α -synuclein is a significant constituent of the amyloid plaques in AD. This 35-amino acid peptide, known as NAC, was shown to amount to about 10% of the amyloid plaque. In fact, recent studies suggest that a very significant fraction of AD patients also have α -synuclein pathology (Arai *et al.*, 2001). This indicates that α -synuclein is a key player in the pathogenesis of

several neurodegenerative disorders (Goedert, 2001a). The fibrillogenesis of α -synuclein *in vitro* has been studied extensively. In particular, accumulated data strongly suggest that the formation of a partially folded intermediate (possessing the major characteristics of the premolten globule) represents the critical first step of α -synuclein fibrillogenesis. This intermediate can be stabilized by numerous factors, including high temperatures (Uversky *et al.*, 2001a), low pH (Uversky *et al.*, 2001a), the presence of several common pesticides and herbicides (Uversky *et al.*, 2001c, 2002; Manning-Bog *et al.*), or metal ions (Uversky *et al.*, 2001b, 2002), or at moderate concentrations of TMAO (Uversky *et al.*, 2001d), or other organic solvents (Munishkina *et al.*, 2003). Under all these conditions α -synuclein was shown to fibrillate very efficiently.

4.2.4. Amylin

In addition to insulin, pancreatic islet β -cells produce a peptide called amylin or islet amyloid polypeptide, IAAP (Cooper *et al.*, 1987). Amylin has several functions associated with the normal regulation of energy metabolism. Dysfunction of amylin due to mutation and/or amyloid fibril formation has been associated with the development of noninsulin-dependent diabetes mellitus (NIDDM) (Higham *et al.*, 2000; Jaikaran *et al.*, 2001; Jaikaran and Clark, 2001). Amylin is an unstructured peptide hormone of 37 amino acid residues (see Table 1-1), which was shown to form fibrils under physiological conditions (Goldsbury *et al.*, 2000). Interestingly, the peptide showed formation of a partially folded (premolten globule-like) intermediate early in the fibrillation process (Goldsbury *et al.*, 2000).

4.2.5. Prothymosin α

Prothymosin α (pT α) is 109-residue protein, which is very acidic, containing ~50% aspartic and glutamic acid, no aromatic or cysteine residues, and very few large hydrophobic aliphatic amino acids (Gast *et al.*, 1995). Because of these features, pT α adopts a random coil-like conformation with no regular secondary structure *in vitro* (Gast *et al.*, 1995; Uversky *et al.*, 1999a) (Table 1-1). However, at acidic pH pT α adopts a partially folded conformation (Uversky *et al.*, 1999a). Interestingly, it has been recently shown that at low pH (below pH 3, i.e., under conditions favoring the formation of the premolten globule-like conformation) (Uversky *et al.*, 1999a), pT α is capable of fast formation of regular elongated fibrils with a flat ribbon-like structure 4–5 nm in height and 12–13 nm in width (Pavlov *et al.*, 2002).

4.3. Conformational Prerequisites for Amyloidogenesis: Why a Premolten Globule?

The data on the structural analysis of the early events during the fibrillation of several proteins and polypeptides have demonstrated the critical role of substantially unfolded conformations as fibril precursors. The question then arises as to the nature of these amyloidogenic conformations. Globular proteins under equilibrium conditions may have at least two different partially folded conformations, the molten globule and its precursor, the premolten globule (Fink, 1995a, 1995b; Ptitsyn, 1995; Ptitsyn *et al.*, 1995; Uversky, 1997). Potentially, either of these partially folded conformations (the molten globule or the premolten globule) may play a role as the crucial fibrillation-prone intermediate. However, data accumulated so far are consistent with the assumption that the amyloidogenic species is significantly unfolded, and structurally closer to the premolten globule than to the molten globule state (Uversky and Fink, 2004). The next important questions are: Why a premolten globule? What is so specific about this conformation determining its fate of being the amyloidogenic intermediate?

What is the molecular basis nudging the premolten globule to form extensive intermolecular contacts? The answer to all of these questions is simple and originates from the concept of packing defects, underwrapped hydrogen bonds, discussed in Section 3 of this Chapter. It has been pointed out that there is a dramatic difference between nonamyloidogenic and fibrillation-prone proteins in their amounts of the underwrapped hydrogen bonds, with ~3% and ~50% of the backbone hydrogen bonds being underwrapped in the hemoglobin (a good backbone wrapper) and in ordered region of the human cellular prion protein (a poor backbone wrapper), respectively. On the other hand, the inability to properly wrap a structure intramolecularly is compensated by protein complexation (aggregation) (Fernandez, 2002; Fernandez and Scheraga, 2003; Fernandez *et al.*, 2003). Thus, an important structural prerequisite for amyloidogenesis is the presence of a substantial amount of the underwrapped backbone hydrogen bonds (50% and more). Among different partially folded intermediates, this condition is achievable only in the premolten globule state, a relatively swollen conformation, which lacks globular structure and possesses considerably depleted secondary structure content (~50% or less of the corresponding native value) (Uversky, 2003).

It is important to note that the formation of amyloid-like fibrils does not represent the only pathological hallmark of “conformational” or protein deposition diseases. In several neurodegenerative disorders (as well as in numerous *in vitro* experiments) the protein depositions are composed of amorphous aggregates, cloud-like inclusions without defined structure. Similarly, soluble oligomers represent another alternative final product of the aggregation process. The choice between three aggregation pathways, fibrillation, amorphous aggregate formation, or oligomerization is determined by the amino acid sequence (could be modified by mutations) and by the peculiarities of the protein environment. **Figure 1-1; see color insert** represents a simplified model of protein aggregation and illustrates the idea that aggregation is an extremely complex process, which can be divided into three major steps. The model shows that proteins with different types of structure (α -helical, β -structural, natively unfolded, $\alpha\beta$ or α/β ; single-domain or multidomain; etc., marked on the picture as structures 1a–1e, see **Figure 1-1; see color insert**) are equally subjected to aggregation (Merlini and Bellotti, 2003; Uversky and Fink, 2004). The structural transformation of these diverse soluble proteins into the “sticky” aggregation-prone precursor or intermediate (marked as 2) represents a first stage of the aggregation process. As this intermediate plays a crucial role in the process, it is presented as an enlarged image. The unifying structure of the intermediate is shown for the sake of brevity only. In fact, these aggregation-prone intermediates would be structurally different for different proteins. Furthermore, an intermediate might contain different amounts of ordered structure even for the same protein undergoing different aggregation processes. Overall, we believe that the precursor of soluble aggregates is the most structured, whereas amyloid fibrils are formed from the least ordered conformation [cf. (Khurana *et al.*, 2001)]. It has been also pointed out that the variations in the amount of the ordered structure in the amyloidogenic precursor might be responsible for the formation of fibrils with distinct morphologies (Smith *et al.*, 2003).

The formation of different oligomers (**Figure 1-1; see color insert**, protofibrils, 3a; or protoaggregates, 3b) represents a second stage, which is usually considered as a nucleation step (Merlini and Bellotti, 2003). Initially, the conditions do not favor aggregation, and this initial time corresponds to the lag period that precedes the formation of aggregates. However, once a critical nucleus has been generated, the conditions change in the favor of aggregation with very fast kinetics (Merlini and Bellotti, 2003). As a result, any available aggregation-prone conformation quickly becomes entrapped in the fibrils, 4a; soluble oligomer, 4b; or amorphous aggregate, 4c (**Figure 1-1; see color insert**).

Another important point to remember is that there are several investigations favoring the idea that the deposited proteinaceous inclusions (such as senile plaques in AD brains or Lewy bodies or Lewy neurites in PD brains) are not toxic, but the formation of some protofibrillar structures are responsible for the toxicity (Selkoe, 1997; Mucke *et al.*, 2000; Lashuel *et al.*, 2002a, 2002b; Urbanc

et al., 2002). However, all these issues (multiple pathways of aggregation and problems of cytotoxicity of protein aggregates) are outside the primary scope of this review, which is devoted to the idea that the appearance of the partially folded conformation is a critical early stage of fibrillogenesis and precedes the appearance of any aggregated material.

5. Concluding Remarks

The inability to protect the backbone hydrogen bonds intramolecularly by surrounding the electrostatically interactive amide-carbonyl pair with nonpolar groups from side chains represents an important structural defect, determining the behavior of the protein in solution, its interactivity, and amyloidogenicity. These wrapping deficiencies become indicative of protein aggregation when they are present at high concentrations. Some proteins possess a sufficient amount of underwrapped hydrogen bonds in their native state and, thus, are able to form amyloid fibrils under physiological conditions. The majority of globular proteins has to be considerably destabilized to become amyloidogenic. Substantial data now indicate that relatively unfolded and highly flexible conformations, notably distinct from the rigid globular, molten globule-like, and completely unfolded states, are key species in the fibrillation process. As comparable aggregation behavior has been observed for both globular and natively unfolded proteins, both disease-associated and disease-unrelated polypeptides, we assume that such relatively unfolded conformations are a structural prerequisite for fibril formation in all systems.

6. Abbreviations

AA	Reactive systemic amyloidosis
A β	Amyloid β -protein
AD	Alzheimer's disease
AL	Light chain amyloidosis
α -LA	α -Lactalbumin
ANS	1-Anilino-8-naphthalene sulfonate
FAP	Familial amyloid polyneuropathy
IAAP	Islet amyloid polypeptide
NAC	Nonamyloid component
NIDDM	Noninsulin-dependent diabetes mellitus
NMR	Nuclear magnetic resonance
PD	Parkinson's disease
PDB	Protein data bank
SAA	Serum amyloid A
TMAO	Trimethylamine <i>N</i> -oxide
TTR	Transthyretin

References

- Alonso, A.C., Grundke-Iqbal, I., and Iqbal, K. (1996). Alzheimer's disease hyperphosphorylated tau sequesters normal tau into tangles of filaments and disassembles microtubules. *Nat. Med.* 2:783–787.
- Anfinsen, C.B. (1973). Principles that govern the folding of protein chains. *Science* 181:223–230.

- Arai, Y., Yamazaki, M., Mori, O., Muramatsu, H., Asano, G., and Katayama, Y. (2001). Ialpha-Synuclein-positive structures in cases with sporadic Alzheimer's disease: morphology and its relationship to tau aggregation. *Brain Res.* 888:287–296.
- Avbelj, F., and Baldwin, R.L. (2002). Role of backbone solvation in determining thermodynamic beta propensities of the amino acids. *Proc. Natl. Acad. Sci. USA* 99:1309–1313.
- Baures, P.W., Peterson, S.A., and Kelly, J.W. (1998). Discovering transthyretin amyloid fibril inhibitors by limited screening. *Bioorg. Med. Chem.* 6:1389–1401.
- Becker, J.W., and Reeke, G.N., Jr. (1985). Three-dimensional structure of beta 2-microglobulin. *Proc. Natl. Acad. Sci. USA* 82:4225–4229.
- Bellotti, V., Mangione, P., and Stoppini, M. (1999). Biological activity and pathological implications of misfolded proteins. *Cell. Mol. Life Sci.* 55:977–991.
- Bellotti, V., Mangione, P., and Merlini, G. (2000). Review: immunoglobulin light chain amyloidosis—the archetype of structural and pathogenic variability. *J. Struct. Biol.* 130:280–289.
- Brange, J., Andersen, L., Laursen, E.D., Meyn, G., and Rasmussen, E. (1997). Toward understanding insulin fibrillation. *J. Pharm. Sci.* 86:517–525.
- Bryant, C., Spencer, D.B., Miller, A., Bakaysa, D.L., McCune, K.S., Maple, S.R., Pekar, A.H., and Brems, D.N. (1993). Acid stabilization of insulin. *Biochemistry* 32:8075–8082.
- Buxbaum, J. (1992). Mechanisms of disease: monoclonal immunoglobulin deposition. Amyloidosis, light chain deposition disease, and light and heavy chain deposition disease. *Hematol. Oncol. Clin. North Am.* 6:323–346.
- Canet, D., Sunde, M., Last, A.M., Miranker, A., Spencer, A., Robinson, C.V., and Dobson, C.M. (1999). Mechanistic studies of the folding of human lysozyme and the origin of amyloidogenic behavior in its disease-related variants. *Biochemistry* 38:6419–6427.
- Canet, D., Doering, K., Dobson, C.M., and Dupont, Y. (2001). High-sensitivity fluorescence anisotropy detection of protein-folding events: application to alpha-lactalbumin. *Biophys. J.* 80:1996–2003.
- Chamberlain, A.K., Handel, T.M., and Marqusee, S. (1996). Detection of rare partially folded molecules in equilibrium with the native conformation of RNaseH. *Nat. Struct. Biol.* 3:782–787.
- Chiti, F., Webster, P., Taddei, N., Clark, A., Stefani, M., Ramponi, G., and Dobson, C.M. (1999). Designing conditions for in vitro formation of amyloid protofilaments and fibrils. *Proc. Natl. Acad. Sci. USA* 96:3590–3594.
- Chiti, F., Taddei, N., Stefani, M., Dobson, C.M., and Ramponi, G. (2001). Reduction of the amyloidogenicity of a protein by specific binding of ligands to the native conformation. *Protein Sci.* 10:879–886.
- Cooper, G.J., Willis, A.C., Clark, A., Turner, R.C., Sim, R.B., and Reid, K.B. (1987). Purification and characterization of a peptide from amyloid-rich pancreases of type 2 diabetic patients. *Proc. Natl. Acad. Sci. USA* 84:8628–8632.
- Crowther, R.A., and Goedert, M. (2000). Abnormal tau-containing filaments in neurodegenerative diseases. *J. Struct. Biol.* 130:271–279.
- Delacourte, A., and Buee, L. (1997). Normal and pathological tau proteins as factors for microtubule assembly. *Int. Rev. Cytol.* 171:167–224.
- Dobson, C.M. (1999). Protein misfolding, evolution and disease. *Trends Biochem. Sci.* 24:329–332.
- Dobson, C.M. (2001). The structural basis of protein folding and its links with human disease. *Philos. Trans. R. Soc. Lond B Biol. Sci.* 356:133–145.
- Dobson, C.M. (2002). Protein-misfolding diseases: getting out of shape. *Nature* 418:729–730.
- Dunker, A.K., Lawson, J.D., Brown, C.J., Williams, R.M., Romero, P., Oh, J.S., Oldfield, C.J., Campen, A.M., Ratliff, C.M., Hipps, K.W., Ausio, J., Nissen, M.S., Reeves, R., Kang, C., Kissinger, C.R., Bailey, R.W., Griswold, M.D., Chiu, W., Garner, E.C., and Obradovic, Z. (2001). Intrinsically disordered protein. *J. Mol. Graph. Model.* 19: 26–59.
- Dunker, A.K., Brown, C.J., and Obradovic, Z. (2002). Identification and functions of usefully disordered proteins. *Adv. Protein Chem.* 62:25–49.
- Dyson, H.J., and Wright, P.E. (2002). Coupling of folding and binding for unstructured proteins. *Curr. Opin. Struct. Biol.* 12:54–60.
- Ehrlich, J., and Ratner, I.M. (1961). Amyloidosis of the islets of Langerhans. A restudy of islet in diabetic and non-diabetic individuals. *Am. J. Pathol.* 38:49–59.
- Englander, S.W., and Kallenbach, N.R. (1983). Hydrogen exchange and structural dynamics of proteins and nucleic acids. *Q. Rev. Biophys* 16:521–655.
- Fandrich, M., Fletcher, M.A., and Dobson, C.M. (2001). Amyloid fibrils from muscle myoglobin. *Nature* 410:165–166.
- Fernandez, A. (2001). Conformation-dependent environments in folding proteins. *J. Chem. Phys.* 114:2489–2502.
- Fernandez, A. (2002). Insufficient hydrogen-bond desolvation and prion-related disease. *Eur. J. Biochem.* 269: 4165–4168.

- Fernandez, A. (2004). Packing defects in folded proteins: what cannot be kept dry must be conserved. *J. Mol. Biol.* 337:477–483.
- Fernandez, A., and Berry, R.S. (2003). Proteins with H-bond packing defects are highly interactive with lipid bilayers: implications for amyloidogenesis. *Proc. Natl. Acad. Sci. USA* 100:2391–2396.
- Fernandez, A., and Scheraga, H.A. (2003). Insufficiently dehydrated hydrogen bonds as determinants of protein interactions. *Proc. Natl. Acad. Sci. USA* 100:113–118.
- Fernandez, A., and Scott, L.R. (2003a). Adherence of packing defects in soluble proteins. *Phys. Rev. Lett* 91: 018102–018106.
- Fernandez, A., and Scott, L.R. (2003b). Under-wrapped soluble proteins as signals triggering membrane morphology. *J. Chem. Phys.* 119:6911–6915.
- Fernandez, A., and Scott, R. (2003c). Dehydron: a structurally encoded signal for protein interaction. *Biophys. J.* 85: 1914–1928.
- Fernandez, A., Sosnick, T.R., and Colubri, A. (2002). Dynamics of hydrogen bond desolvation in protein folding. *J. Mol. Biol.* 321:659–675.
- Fernandez, A., Kardos, J., Scott, L.R., Goto, Y., and Berry, R.S. (2003). Structural defects and the diagnosis of amyloidogenic propensity. *Proc. Natl. Acad. Sci. USA* 100:6446–6451.
- Fernandez, A., Scott, L.R., and Berry, R.S. (2004). The nonconserved wrapping of conserved protein folds defines a trend towards increasing connectivity in proteomic networks. *Proc. Natl. Acad. Sci. USA* 101:2823–2827.
- Fink, A.L. (1995a). Compact intermediate states in protein folding. *Annu. Rev. Biophys. Biomol. Struct.* 24:495–522.
- Fink, A.L. (1995b). Molten globules. *Methods Mol. Biol.* 40:343–360.
- Fink, A.L. (1998). Protein aggregation: folding aggregates, inclusion bodies and amyloid. *Fold. Des.* 3:9–15.
- Gast, K., Damaschun, H., Eckert, K., Schulze-Forster, K., Maurer, H.R., Muller-Frohne, M., Zirwer, D., Czarnecki, J., and Damaschun, G. (1995). Prothymosin alpha: a biologically active protein with random coil conformation. *Biochemistry* 34:13211–13218.
- Giasson, B.I., Duda, J.E., Quinn, S.M., Zhang, B., Trojanowski, J.Q., and Lee, V.M. (2002). Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. *Neuron* 34: 521–533.
- Goedert, M. (2001a). Parkinson's disease and other alpha-synucleinopathies. *Clin. Chem. Lab Med.* 39:308–312.
- Goedert, M. (2001b). The significance of tau and alpha-synuclein inclusions in neurodegenerative diseases. *Curr. Opin. Genet. Dev.* 11:343–351.
- Goers, J., Permyakov, S.E., Permyakov, E.A., Uversky, V.N., and Fink, A.L. (2002). Conformational prerequisites for alpha-lactalbumin fibrillation. *Biochemistry* 41:12546–12551.
- Goldsbury, C., Goldie, K., Pellaud, J., Seelig, J., Frey, P., Muller, S.A., Kistler, J., Cooper, G.J.S., and Aebi, U. (2000). Amyloid fibril formation from full-length and fragments of amylin. *J. Struct. Biol.* 130:352–362.
- Guijarro, J.I., Sunde, M., Jones, J.A., Campbell, I.D., and Dobson, C.M. (1998). Amyloid fibril formation by an SH3 domain. *Proc. Natl. Acad. Sci. USA* 95:4224–4228.
- Hagstedt, T., Lichtenberg, B., Wille, H., Mandelkow, E.M., and Mandelkow, E. (1989). Tau-protein becomes long and stiff upon phosphorylation—correlation between paracrystalline structure and degree of phosphorylation. *J. Cell Biol.* 109:1643–1651.
- Hamada, D., and Dobson, C.M. (2002). A kinetic study of beta-lactoglobulin amyloid fibril formation promoted by urea. *Protein Sci.* 11:2417–2426.
- Hammarstrom, P., Wiseman, R.L., Powers, E.T., and Kelly, J.W. (2003). Prevention of transthyretin amyloid disease by changing protein misfolding energetics. *Science* 299:713–716.
- Heegaard, N.H., Sen, J.W., Kaarsholm, N.C., and Nissen, M.H. (2001). Conformational intermediate of the amyloidogenic protein beta 2-microglobulin at neutral pH. *J. Biol. Chem.* 276:32657–32662.
- Higham, C.E., Jaikaran, E.T., Fraser, P.E., Gross, M., and Clark, A. (2000). Preparation of synthetic human islet amyloid polypeptide (IAPP) in a stable conformation to enable study of conversion to amyloid-like fibrils. *FEBS Lett.* 470:55–60.
- Himmler, A. (1989). Structure of the bovine tau-gene—alternatively spliced transcripts generate A protein family. *Mol. Cell. Biol.* 9:1389–1396.
- Hong, D.P., Gozu, M., Hasegawa, K., Naiki, H., and Goto, Y. (2002). Conformation of beta(2)-microglobulin amyloid fibrils analyzed by reduction of the disulfide bond. *J. Biol. Chem.* 277:21554–21560.
- Hoshino, M., Katou, H., Hagihara, Y., Hasegawa, K., Naiki, H., and Goto, Y. (2002). Mapping the core of the beta(2)-microglobulin amyloid fibril by H/D exchange. *Nat. Struct. Biol.* 9:332–336.
- Hurle, M.R., Helms, L.R., Li, L., Chan, W., and Wetzel, R. (1994). A role for destabilizing amino acid replacements in light-chain amyloidosis. *Proc. Natl. Acad. Sci. USA* 91:5446–5450.

- Iakoucheva, L.M., Brown, C.J., Lawson, J.D., Obradovic, Z., and Dunker, A.K. (2002). Intrinsic disorder in cell-signaling and cancer-associated proteins. *J. Mol. Biol.* 323:573–584.
- Jadoul, M., Garbar, C., Noel, H., Sennesael, J., Vanholder, R., Bernaert, P., Rorive, G., Hanique, G., and van Ypersele, D.S. (1997). Histological prevalence of beta 2-microglobulin amyloidosis in hemodialysis: a prospective post-mortem study. *Kidney Int.* 51:1928–1932.
- Jaikaran, E.T. and Clark, A. (2001). Islet amyloid and type 2 diabetes: from molecular misfolding to islet pathophysiology. *Biochim. Biophys. Acta* 1537:179–203.
- Jaikaran, E.T., Higham, C.E., Serpell, L.C., Zurdo, J., Gross, M., Clark, A., and Fraser, P.E. (2001). Identification of a novel human islet amyloid polypeptide beta-sheet domain and factors influencing fibrillogenesis. *J. Mol. Biol.* 308:515–525.
- Jiang, X., Smith, C.S., Petrassi, H.M., Hammarstrom, P., White, J.T., Sacchettini, J.C., and Kelly, J.W. (2001). An engineered transthyretin monomer that is nonamyloidogenic, unless it is partially denatured. *Biochemistry* 40:11442–11452.
- Kayed, R., Bernhagen, J., Greenfield, N., Sweimeh, K., Brunner, H., Voelter, W., and Kapurniotu, A. (1999). Conformational transitions of islet amyloid polypeptide (IAPP) in amyloid formation in vitro. *J. Mol. Biol.* 287:781–796.
- Kelly, J.W. (1996). Alternative conformations of amyloidogenic proteins govern their behavior. *Curr. Opin. Struct. Biol.* 6:11–17.
- Kelly, J.W. (1998). The alternative conformations of amyloidogenic proteins and their multi-step assembly pathways. *Curr. Opin. Struct. Biol.* 8:101–106.
- Kenessey, A., and Yen, S.H.C. (1993). The extent of phosphorylation of fetal-tau is comparable to that of Phf-tau from Alzheimer paired helical filaments. *Brain Res.* 629:40–46.
- Khurana, R., Gillespie, J.R., Talapatra, A., Minert, L.J., Ionescu-Zanetti, C., Millett, I., and Fink, A.L. (2001). Partially folded intermediates as critical precursors of light chain amyloid fibrils and amorphous aggregates. *Biochemistry* 40:3525–3535.
- Kim, Y., Wall, J.S., Meyer, J., Murphy, C., Randolph, T.W., Manning, M.C., Solomon, A., and Carpenter, J.F. (2000). Thermodynamic modulation of light chain amyloid fibril formation. *J. Biol. Chem.* 275:1570–1574.
- Kirkitadze, M.D., Condrón, M.M., and Teplow, D.B. (2001). Identification and characterization of key kinetic intermediates in amyloid beta-protein fibrillogenesis. *J. Mol. Biol.* 312:1103–1119.
- Klabunde, T., Petrassi, H.M., Oza, V.B., Raman, P., Kelly, J.W., and Sacchettini, J.C. (2000). Rational design of potent human transthyretin amyloid disease inhibitors. *Nat. Struct. Biol.* 7:312–321.
- Konno, T., Murata, K., and Nagayama, K. (1999). Amyloid-like aggregates of a plant protein: a case of a sweet-tasting protein, monellin. *Febs Lett.* 454:122–126.
- Krebs, M.R., Wilkins, D.K., Chung, E.W., Pitkeathly, M.C., Chamberlain, A.K., Zurdo, J., Robinson, C.V., and Dobson, C.M. (2000). Formation and seeding of amyloid fibrils from wild-type hen lysozyme and a peptide fragment from the beta-domain. *J. Mol. Biol.* 300:541–549.
- Lai, Z., Colón, W., and Kelly, J.W. (1996). The acid-mediated denaturation pathway of transthyretin yields a conformational intermediate that can self-assemble into amyloid. *Biochemistry* 35:6470–6482.
- Lansbury, P.T., Jr. (1999). Evolution of amyloid: what normal protein folding may tell us about fibrillogenesis and disease. *Proc. Natl. Acad. Sci. USA* 96:3342–3344.
- Lashuel, H.A., Wurth, C., Woo, L., and Kelly, J.W. (1999). The most pathogenic transthyretin variant, L55P, forms amyloid fibrils under acidic conditions and protofilaments under physiological conditions. *Biochemistry* 38:13560–13573.
- Lashuel, H.A., Hartley, D., Petre, B.M., Walz, T., and Lansbury, P.T., Jr. (2002a). Neurodegenerative disease: amyloid pores from pathogenic mutations. *Nature* 418:291.
- Lashuel, H.A., Petre, B.M., Wall, J., Simon, M., Nowak, R.J., Walz, T., and Lansbury, P.T., Jr. (2002b). Alpha-synuclein, especially the Parkinson's disease-associated mutants, forms pore-like annular and tubular protofibrils. *J. Mol. Biol.* 322:1089–1102.
- Lee, M.K., Stirling, W., Xu, Y.Q., Xu, X.Y., Qui, D., Mandir, A.S., Dawson, T.M., Copeland, N.G., Jenkins, N.A., and Price, D.L. (2002). Human alpha-synuclein-harboring familial Parkinson's disease-linked Ala-53 → Thr mutation causes neurodegenerative disease with alpha-synuclein aggregation in transgenic mice. *Proc. Natl. Acad. Sci. USA* 99:8968–8973.
- Link, C.D. (2001). Transgenic invertebrate models of age-associated neurodegenerative diseases. *Mech. Ageing Dev.* 122:1639–1649.
- Litvinovich, S.V., Brew, S.A., Aota, S., Akiyama, S.K., Haudenschild, C., and Ingham, K.C. (1998). Formation of amyloid-like fibrils by self-association of a partially unfolded fibronectin type III module. *J. Mol. Biol.* 280:245–258.
- Manning-Bog, A.B., McCormack, A.L., Li, J., Uversky, V.N., Fink, A.L., and Di Monte, D.A. (2002). The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice. *J. Biol. Chem.* 277:1641–1644.

- Marhaug, G., and Dowton, S.B. (1994). Serum amyloid A: an acute phase apolipoprotein and precursor of AA amyloid. *Baillieres Clin. Rheumatol.* 8:553–573.
- Masliah, E., Rockenstein, E., Veinbergs, I., Mallory, M., Hashimoto, M., Takeda, A., Sagara, Y., Sisk, A., and Mucke, L. (2000). Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. *Science* 287:1265–1269.
- McCammon, M.G., Scott, D.J., Keetch, C.A., Greene, L.H., Purkey, H.E., Petrassi, H.M., Kelly, J.W., and Robinson, C.V. (2002). Screening transthyretin amyloid fibril inhibitors: characterization of novel multiprotein, multiligand complexes by mass spectrometry. *Structure (Camb.)* 10:851–863.
- McCubbin, W.D., Kay, C.M., Narindrasorasak, S., and Kisilevsky, R. (1988). Circular-dichroism studies on two murine serum amyloid A proteins. *Biochem. J.* 256:775–783.
- McParland, V.J., Kad, N.M., Kalverda, A.P., Brown, A., Kirwin-Jones, P., Hunter, M.G., Sunde, M., and Radford, S.E. (2000). Partially unfolded states of beta(2)-microglobulin and amyloid formation in vitro. *Biochemistry* 39:8735–8746.
- McParland, V.J., Kalverda, A.P., Homans, S.W., and Radford, S.E. (2002). Structural properties of an amyloid precursor of beta(2)-microglobulin. *Nat. Struct. Biol.* 9:326–331.
- Merlini, G., and Bellotti, V. (2003). Molecular mechanisms of amyloidosis. *N. Engl. J. Med.* 349:583–596.
- Morishimakawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Yoshida, H., Watanabe, A., Titani, K., and Ihara, Y. (1995). Hyperphosphorylation of tau in Phf. *Neurobiol. Aging* 16:365–371.
- Morozova-Roche, L.A., Zurdo, J., Spencer, A., Noppe, W., Receveur, V., Archer, D.B., Joniau, M., and Dobson, C.M. (2000). Amyloid fibril formation and seeding by wild-type human lysozyme and its disease-related mutational variants. *J. Struct. Biol.* 130:339–351.
- Mucke, L., Masliah, E., Yu, G.Q., Mallory, M., Rockenstein, E.M., Tatsuno, G., Hu, K., Kholodenko, D., Johnson-wood, K., and McConlogue, L. (2000). High-level neuronal expression of abeta 1–42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J. Neurosci.* 20:4050–4058.
- Munishkina, L.A., Phelan, C., Uversky, V.N., and Fink, A.L. (2003). Conformational behavior and aggregation of alpha-synuclein in organic solvents: modeling the effects of membranes. *Biochemistry* 42:2720–2730.
- Nielsen, L., Frokjaer, S., Brange, J., Uversky, V.N., and Fink, A.L. (2001a). Probing the mechanism of insulin fibril formation with insulin mutants. *Biochemistry* 40:8397–8409.
- Nielsen, L., Khurana, R., Coats, A., Frokjaer, S., Brange, J., Vyas, S., Uversky, V.N., and Fink, A.L. (2001b). Effect of environmental factors on the kinetics of insulin fibril formation: elucidation of the molecular mechanism. *Biochemistry* 40:6036–6046.
- Oza, V.B., Petrassi, H.M., Purkey, H.E., and Kelly, J.W. (1999). Synthesis and evaluation of anthranilic acid-based transthyretin amyloid fibril inhibitors. *Bioorg. Med. Chem. Lett.* 9:1–6.
- Oza, V.B., Smith, C., Raman, P., Koepf, E.K., Lashuel, H.A., Petrassi, H.M., Chiang, K.P., Powers, E.T., Sacchettini, J., and Kelly, J.W. (2002). Synthesis, structure, and activity of diclofenac analogues as transthyretin amyloid fibril formation inhibitors. *J. Med. Chem.* 45:321–332.
- Pavlov, N.A., Cherny, D.I., Heim, G., Jovin, T.M., and Subramaniam, V. (2002). Amyloid fibrils from the mammalian protein prothymosin alpha. *FEBS Lett.* 517:37–40.
- Pepys, M.B., Hawkins, P.N., Booth, D.R., Vigushin, D.M., Tennent, G.A., Soutar, A.K., Totty, N., Nguyen, O., Blake, C.C., and Terry, C.J. (1993). Human lysozyme gene mutations cause hereditary systemic amyloidosis. *Nature* 362:553–557.
- Permyakov, E.A., and Berliner, L.J. (2000). alpha-Lactalbumin: structure and function. *FEBS Lett.* 473:269–274.
- Pertinhez, T.A., Bouchard, M., Tomlinson, E.J., Wain, R., Ferguson, S.J., Dobson, C.M., and Smith, L.J. (2001). Amyloid fibril formation by a helical cytochrome. *FEBS Lett.* 495:184–186.
- Peterson, S.A., Klabunde, T., Lashuel, H.A., Purkey, H., Sacchettini, J.C., and Kelly, J.W. (1998). Inhibiting transthyretin conformational changes that lead to amyloid fibril formation. *Proc. Natl. Acad. Sci. USA* 95:12956–12960.
- Prusiner, S.B. (1998). Prions. *Proc. Natl. Acad. Sci. USA* 95:13363–13383.
- Ptitsyn, O.B. (1995). Molten globule and protein folding. *Adv. Protein Chem.* 47:83–229.
- Ptitsyn, O.B., Bychkova, V.E., and Uversky, V.N. (1995). Kinetic and equilibrium folding intermediates. *Philos. Trans. R. Soc. Lond B Biol. Sci* 348:35–41.
- Raffen, R., Dieckman, L.J., Szpunar, M., Wunschl, C., Pokkuluri, P.R., Dave, P., Wilkins, S.P., Cai, X., Schiffer, M., and Stevens, F.J. (1999). Physicochemical consequences of amino acid variations that contribute to fibril formation by immunoglobulin light chains. *Protein Sci.* 8:509–517.
- Raghu, P., Reddy, G.B., and Sivakumar, B. (2002). Inhibition of transthyretin amyloid fibril formation by 2,4-dinitrophenol through tetramer stabilization. *Arch. Biochem. Biophys.* 400:43–47.

- Ramirez-Alvarado, M., Merkel, J.S., and Regan, L. (2000). A systematic exploration of the influence of the protein stability on amyloid fibril formation in vitro. *Proc. Natl. Acad. Sci. USA* 97:8979–8984.
- Richardson, J.S., and Richardson, D.C. (2002). Natural beta-sheet proteins use negative design to avoid edge-to-edge aggregation. *Proc. Natl. Acad. Sci. USA* 99:2754–2759.
- Rochet, J.C., and Lansbury, P.T., Jr. (2000). Amyloid fibrillogenesis: themes and variations. *Curr. Opin. Struct. Biol.* 10:60–68.
- Saraiva, M.J. (2001). Transthyretin amyloidosis: a tale of weak interactions. *FEBS Lett.* 498:201–203.
- Saraiva, M.J., Birken, S., Costa, P.P., and Goodman, D.S. (1984). Amyloid fibril protein in familial amyloidotic polyneuropathy, Portuguese type. Definition of molecular abnormality in transthyretin (prealbumin). *J. Clin. Invest* 74:104–119.
- Schweers, O., Schonbrunn-Hanebeck, E., Marx, A., and Mandelkow, E. (1994). Structural studies of tau protein and Alzheimer paired helical filaments show no evidence for beta-structure. *J. Biol. Chem.* 269:24290–24297.
- Selkoe, D.J. (1997). Neuroscience: Alzheimer's disease: genotypes, phenotype, and treatments. *Science* 275:630–631.
- Smith, D.P., Jones, S., Serpell, L.C., Sunde, M., and Radford, S.E. (2003). A systematic investigation into the effect of protein destabilisation on beta 2-microglobulin amyloid formation. *J. Mol. Biol.* 330:943–954.
- Souillac, P.O., Uversky, V.N., Millett, I.S., Khurana, R., Doniach, S., and Fink, A.L. (2002a). Effect of association state and conformational stability on the kinetics of immunoglobulin light chain amyloid fibril formation at physiological pH. *J. Biol. Chem.* 277:12657–12665.
- Souillac, P.O., Uversky, V.N., Millett, I.S., Khurana, R., Doniach, S., and Fink, A.L. (2002b). Elucidation of the molecular mechanism during the early events in immunoglobulin light chain amyloid fibrillation: evidence for an off-pathway oligomer at acidic pH. *J. Biol. Chem.* 277:12666–12679.
- Sunde, M., Serpell, L.C., Bartlam, M., Fraser, P.E., Pepys, M.B., and Blake, C.C. (1997). Common core structure of amyloid fibrils by synchrotron X-ray diffraction. *J. Mol. Biol.* 273:729–739.
- Teplow, D.B. (1998). Structural and kinetic features of amyloid beta-protein fibrillogenesis. *Amyloid Int. J. Exp. Clin. Invest.* 5:121–142.
- Tomic, M.T., Somoza, J.R., Wemmer, D.E., Park, Y.W., Cho, J.M., and Kim, S.H. (1992). H-1 resonance assignments, secondary structure and general topology of single-chain monellin in solution as determined by H-1 2D-Nmr. *J. Biomol. NMR* 2:557–572.
- Urbanc, B., Cruz, L., Le, R., Sanders, J., Ashe, K.H., Duff, K., Stanley, H.E., Irizarry, M.C., and Hyman, B.T. (2002). Neurotoxic effects of thioflavin S-positive amyloid deposits in transgenic mice and Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* 99:13990–13995.
- Uversky, V.N. (1997). Diversity of equilibrium compact forms of denatured globular proteins. *Protein Pept. Lett.* 4:355–367.
- Uversky, V.N. (2002a). Natively unfolded proteins: a point where biology waits for physics. *Protein Sci.* 11:739–756.
- Uversky, V.N. (2002b). What does it mean to be natively unfolded? *Eur. J. Biochem.* 269:2–12.
- Uversky, V.N. (2003). Protein folding revisited. A polypeptide chain at the folding-misfolding-nonfolding cross-roads: which way to go? *Cell Mol. Life Sci.* 60:1852–1871.
- Uversky, V.N., and Fink, A.L. (2004). Conformational constraints for amyloid fibrillation: the importance of being unfolded. *Biochim. Biophys. Acta* 1698:131–153.
- Uversky, V.N., Gillespie, J.R., Millett, I.S., Khodyakova, A.V., Vasiliev, A.M., Chernovskaya, T.V., Vasilenko, R.N., Kozovskaya, G.D., Dolgikh, D.A., Fink, A.L., Doniach, S., and Abramov, V.M. (1999a). Natively unfolded human prothymosin alpha adopts partially folded collapsed conformation at acidic pH. *Biochemistry* 38:15009–15016.
- Uversky, V.N., Talapatra, A., Gillespie, J.R., and Fink, A.L. (1999b). Protein deposits as the molecular basis of amyloidosis. I. Systemic amyloidoses. *Med. Sci. Monitor* 5:1001–1012.
- Uversky, V.N., Talapatra, A., Gillespie, J.R., and Fink, A.L. (1999c). Protein deposits as the molecular basis of amyloidosis. II. Localized amyloidosis and neurodegenerative disorders. *Med. Sci. Monitor* 5:1238–1254.
- Uversky, V.N., Li, J., and Fink, A.L. (2001a). Evidence for a partially folded intermediate in alpha-synuclein fibril formation. *J. Biol. Chem.* 276:10737–10744.
- Uversky, V.N., Li, J., and Fink, A.L. (2001b). Metal-triggered structural transformations, aggregation, and fibrillation of human alpha-synuclein. A possible molecular link between Parkinson's disease and heavy metal exposure. *J. Biol. Chem.* 276:44284–44296.
- Uversky, V.N., Li, J., and Fink, A.L. (2001c). Pesticides directly accelerate the rate of alpha-synuclein fibril formation: a possible factor in Parkinson's disease. *FEBS Lett.* 500:105–108.

- Uversky, V.N., Li, J., and Fink, A.L. (2001d). Trimethylamine-N-oxide-induced folding of alpha-synuclein. *FEBS Lett.* 509:31–35.
- Uversky, V.N., Li, J., Bower, K., and Fink, A.L. (2002). Synergistic effects of pesticides and metals on the fibrillation of alpha-synuclein: implications for Parkinson's disease. *Neurotoxicology* 23:527–536.
- Uversky, V.N., Winter, S., Galzitskaya, O.V., Kittler, L., and Lober, G. (1998). Hyperphosphorylation induces structural modification of tau-protein. *FEBS Lett.* 439:21–25.
- Vulliet, R., Halloran, S.M., Braun, R.K., Smith, A.J., and Lee, G. (1992). Proline-directed phosphorylation of human tau-protein. *J. Biol. Chem.* 267:22570–22574.
- Wall, J., Murphy, C.L., and Solomon, A. (1999a). In vitro immunoglobulin light chain fibrillogenesis. *Methods Enzymol.* 309:204–217.
- Wall, J., Schell, M., Murphy, C., Hrnčić, R., Stevens, F.J., and Solomon, A. (1999b). Thermodynamic instability of human lambda 6 light chains: correlation with fibrillogenicity. *Biochemistry* 38:14101–14108.
- Weinreb, P.H., Zhen, W., Poon, A.W., Conway, K.A., and Lansbury, P.T., Jr. (1996). NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded. *Biochemistry* 35:13709–13715.
- Weiss, M.A., Nguyen, D.T., Khait, I., Inouye, K., Frank, B.H., Beckage, M., O'Shea, E., Shoelson, S.E., Karplus, M., and Neuringer, L.J. (1989). Two-dimensional NMR and photo-CIDNP studies of the insulin monomer: assignment of aromatic resonances with application to protein folding, structure, and dynamics. *Biochemistry* 28:9855–9873.
- Westermarck, P., and Wilander, E. (1983). Islet amyloid in type-2 (non-insulin-dependent) diabetes is related to insulin. *Diabetologia* 24:342–346.
- Westermarck, P., Wernstedt, C., Wilander, E., Hayden, D.W., O'Brien, T.D., and Johnson, K.H. (1987). Amyloid fibrils in human insulinoma and islets of Langerhans of the diabetic cat are derived from a neuropeptide-like protein also present in normal islet cells. *Proc. Natl. Acad. Sci. USA* 84:3881–3885.
- Westermarck, P., Sletten, K., Johansson, B., and Cornwell, G.G., III (1990). Fibril in senile systemic amyloidosis is derived from normal transthyretin. *Proc. Natl. Acad. Sci. USA* 87:2843–2845.
- Wetzel, R. (1997). Domain stability in immunoglobulin light chain deposition disorders. *Adv. Protein Chem.* 50:183–242.
- Wilkins, J., Gallimore, J.R., Tennent, G.A., Hawkins, P.N., Limburg, P.C., van Rijswijk, M.H., Moore, E.G., and Pepys, M.B. (1994). Rapid automated enzyme immunoassay of serum amyloid A. *Clin. Chem* 40:1284–1290.
- Wright, P.E., and Dyson, H.J. (1999). Intrinsically unstructured proteins: re-assessing the protein structure–function paradigm. *J. Mol. Biol.* 293:321–331.
- Yutani, K., Takayama, G., Goda, S., Yamagata, Y., Maki, S., Namba, K., Tsunasawa, S., and Ogasahara, K. (2000). The process of amyloid-like fibril formation by methionine aminopeptidase from a hyperthermophile, *Pyrococcus furiosus*. *Biochemistry* 39:2769–2777.
- Zerovnik, E. (2002). Amyloid-fibril formation. Proposed mechanisms and relevance to conformational disease. *Eur. J. Biochem.* 269:3362–3371.

The Generic Nature of Protein Folding and Misfolding

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1. Abstract

The ability of proteins to fold to their functional states is an astonishing example of the power of biological evolution at the molecular level. Despite the large number of different native protein folds, the process of folding can be described in terms of a universal mechanism that appears to be based on the generation of the correct overall topology through interactions involving a relatively small number of residues. Protein misfolding is an intrinsic aspect of normal folding within the complex cellular environment, and its effects are minimized in living systems by the action of a range of protective mechanisms including molecular chaperones and quality control systems. Unfolded and misfolded proteins have a tendency to aggregate to form a variety of species including the highly organized and kinetically stable amyloid fibrils. The latter species represent a generic form of structure resulting from the inherent polymer properties of polypeptide chains, and their formation is associated with a wide range of debilitating human diseases. Amyloid fibrils and their precursors appear to have similar adverse effects on cellular function regardless of the sequence of the component peptide or protein. Our increasing knowledge of the interplay between different forms of protein structure and their generic characteristics provides a platform for rational therapeutic intervention designed to prevent or treat this whole family of diseases.

2. Introduction

Virtually every chemical process on which our lives depend is stimulated or controlled by protein molecules (Branden and Tooze, 1999). Different proteins are distinguished by a different order of amino acids in the polymeric sequence of typically 300 such building blocks. Following their synthesis in the cell, the majority of proteins must be converted into tightly folded compact structures in order to function. As many of these structures are astonishingly intricate, the fact that folding is usually extremely efficient is a remarkable testament to the power of evolutionary biology. Although proteins are the most abundant molecules in living systems other than water, the 100,000 or so different types of proteins within our bodies represent only a tiny fraction of all possible sequences. Indeed, as there are 20 different naturally occurring amino acids, the total possible number of different proteins with the average size of those in our bodies is astronomical, much greater than the number of atoms in the universe. Moreover, the properties of natural proteins are not typical of random sequences, but have been selected through evolutionary pressure to have specific characteristics—of which the ability to fold to unique structures and hence to generate enormous selectivity and diversity in their functions—is a particularly important one. As we shall see later, however, under

some conditions even natural proteins can revert to behavior that is typical of polymers that have not been subject to such careful evolutionary selection.

The interior of a cell is a highly crowded environment in which proteins and other macromolecules are present at a concentration that can exceed 300mg/ml (Ellis and Minton, 2003). Within the cells of all living organisms, however, there is an array of auxiliary factors that assist in the folding process, including folding catalysts and molecular chaperones (Hartl and Hayer-Hartl, 2002). These factors serve to enable polypeptide chains to fold efficiently in the complex milieu of the cell, but they do not determine their native structures; the latter are fully encoded by the amino acid sequences. How proteins find their unique native states from the information contained within their sequences is a question at the heart of molecular biology. Furthermore, the reproducibility and complexity of biological self-assembly compared to related processes in nonbiological systems is arguably the most remarkable feature of living systems. Understanding protein folding, perhaps the most fundamental example of biological self-assembly, is therefore a first step on the path to resolving one of the most important questions that can be addressed by modern science (Vendruscolo *et al.*, 2003).

3. The Universal Mechanism of Protein Folding

It is only recently that the mechanism by which even the simplest of proteins fold to specific structures has been defined in any detail. There is strong evidence that the native state of a protein corresponds, except in very rare circumstances, to the structure that is the most stable under physiological conditions. Nevertheless, the total number of possible conformations of a polypeptide chain is so large that it would take an enormous length of time to find this particular structure through a systematic search of all conformational space. Recent experimental and theoretical studies have, however, provided a resolution of this problem (Wolynes *et al.*, 1995; Karplus, 1997; Dill and Chan, 1997; Dobson *et al.*, 1998; Dobson, 2003). It is now clear that the folding of a small protein does not involve a series of mandatory steps between well-defined partially folded states, but rather a stochastic search of the many conformations accessible to a polypeptide chain. The conceptual basis of such a mechanism is shown in **Figure 2-1; see color insert**. In essence, the inherent fluctuations in the conformation of an incompletely folded polypeptide chain enable even residues at very different positions in the amino acid sequence to come into contact with one other. Because the correct (native-like) interactions between different residues are on average more stable than the incorrect (nonnative) ones, such a search mechanism is, in principle, able to find the lowest energy structure provided that no substantial barriers develop between different conformations (Wolynes *et al.*, 1995; Karplus, 1997; Dill and Chan, 1997; Dobson *et al.*, 1998; Dobson, 2003). It is evident that this process is extremely efficient for those special sequences that have been selected during evolution to fold to globular structures, and indeed, only a very small number of all possible conformations needs be sampled during the search process.

This stochastic description of protein folding involves the concept of an “energy landscape” for each protein, describing the free energy of the polypeptide chain as a function of its conformational properties. To enable a protein to fold efficiently, the landscape required has been likened to a funnel because the conformational space accessible to the polypeptide chain becomes more restricted as the native state is approached (Wolynes *et al.*, 1995; Dill and Chan, 1997). In essence, the high degree of disorder of the polypeptide chain is reduced as folding progresses because the more favorable enthalpy associated with stable native-like interactions can offset the decreasing entropy of the polypeptide chain as the structure becomes more ordered. The exact manner in which the correct overall fold can be achieved through such a process is emerging primarily from studies of a group

of small proteins—most having less than 100 residues—that fold to their native states without populating significantly any intermediate states (Jackson, 1998). A particularly important experimental strategy has been to use site-directed mutagenesis to probe the roles of individual residues in the folding process (Matouschek *et al.*, 1989; Fersht, 1999, 2000; Vendruscolo *et al.*, 2001). The results of a wide range of studies suggest that the fundamental mechanism of folding can be described as “nucleation-condensation,” in which a folding nucleus of a small number of residues forms, about which the remainder of the structure can then condense (Fersht, 2000).

Detailed insight into how such a generic mechanism can generate unique folds for specific sequences of amino acids has come from a combination of experimental and theoretical studies (Dobson and Karplus, 1999; Fersht and Daggett, 2002). The ultimate objective is to describe complete energy landscapes for folding reactions, and to understand exactly how these are defined by the sequences involved. Recently, experimental data have been incorporated directly in computer simulations of folding, and this approach has allowed structural ensembles representing a variety of important states populated during the folding of particular proteins to be defined in atomic detail as Figure 2-1 illustrates (Vendruscolo *et al.*, 2001; Korzhnev *et al.*, 2004; Vendruscolo and Dobson, 2005). Such studies suggest that a crucial aspect of the transition states of folding reactions is that they possess the same overall topology as the native fold (Debe *et al.*, 1999; Vendruscolo *et al.*, 2001; Dobson, 2003; Makarov and Plaxco, 2003; Lindorff-Larsen *et al.*, 2004). It appears that this topology can result from the acquisition of a native-like environment for a group of residues that constitute the core of the folding nucleus; in essence, these interactions force the chain to adopt a rudimentary native-like architecture (Vendruscolo *et al.*, 2001; Lindorff-Larsen *et al.*, 2004; Vendruscolo and Dobson, 2005). Once this topology has been achieved, the native structure is almost invariably generated when the remainder of the protein coalesces around this nucleus. Conversely, if these key interactions are not formed, the protein cannot fold to a stable globular structure. As all the protein molecules have to pass through the transition state region of the energy landscape prior to achieving their folded state, this mechanism therefore acts also as a “quality control” process by which misfolding can generally be avoided (Davis *et al.*, 2002). Although the details of the way different proteins fold may appear to differ dramatically, in terms of the rates of folding and the type of species populated during the folding process, the essential features of this overall mechanism can be considered to be universal. For example, for proteins that have regions of very high helical propensity, this type of structure may be substantially formed early in the folding process. The transition state may then appear to be as a rather diffuse structure in which a relatively large number of less completely formed interactions define the overall topology (Fersht, 2000; Paci *et al.*, 2004). The folding of such proteins may be well described by a specific mechanism such as “diffusion-collision” (Karplus and Weaver, 1994), but can still be viewed as a special case of the generic mechanism by which all proteins fold (Dobson *et al.*, 1998; Dobson, 2003).

Although the *in vitro* folding of small proteins appears to be predominantly two state, the folding of proteins having more than about 100 residues has been found to involve the significant population of a larger number of species than just the highly unfolded and fully folded states (Dobson *et al.*, 1998; Fersht, 1999). Experiments show that the resulting folding intermediates often correspond to species in which segments of the protein have become highly native-like, while others have yet to achieve a folded state. In other cases the protein may have formed a significant proportion of nonnative interactions, and hence, becomes trapped at least transiently in a misfolded state (Dobson *et al.*, 1998; Capaldi *et al.*, 2002). Indeed, it appears that larger proteins generally fold in modules, that is, that folding takes place largely independently in different segments or domains of the protein (Panchenko *et al.*, 1996; Dobson *et al.*, 1998). In such cases, key interactions are likely to define the fold within local regions or domains, and other specific interactions ensure that these initially folded regions subsequently interact appropriately to form the correct overall structure (Vendruscolo and

Dobson, 2005). The fully native structure is only acquired, however, when all the native-like interactions are formed both within and between the domains; this happens in a final cooperative folding step when all the side chains become locked in their unique close-packed arrangement and water is excluded from the protein core (Cheung *et al.*, 2002). Such a mechanism is appealing because it begins to explain that highly complex structures may be assembled in manageable pieces, each of which achieves its overall architecture by the mechanism described above for small proteins. Moreover, such a principle can readily be extended to describe the assembly of complexes containing a variety of different proteins, and in some cases other macromolecules, notably nucleic acids (Trieber and Williamson, 1999). Thus, even large molecular machines such as the ribosome or the proteasome can be assembled efficiently and with high fidelity.

4. Protein Folding and Misfolding in the Cellular Environment

The folding of some proteins *in vivo* appears to be cotranslational, that is, it begins when the nascent chain is still being synthesized on the ribosome (Hardesty and Kramer, 2001). Electron microscopy and X-ray crystallography are now providing remarkably detailed structures of ribosomes in a variety of different states associated with protein synthesis (Yusopov *et al.*, 2001). Moreover, preliminary experiments have suggested that it might soon be possible to observe the conformational properties of polypeptide chains as they emerge during the synthetic process (Woolhead *et al.*, 2004; Gilbert *et al.*, 2004). Other proteins are thought to undergo the major part of their folding in the cytoplasm only after release from the ribosome, while yet others fold in specific compartments such as the endoplasmic reticulum (ER) following translocation through membranes (Jenni and Ban, 2003). Although many details of the folding of particular proteins will depend on the environment in which it takes place, the fundamental principles of folding derived from *in vitro* studies and discussed above are unlikely to be changed in any significant manner. But as incompletely folded chains expose regions of the polypeptide molecule that are buried in the native state, such species are prone to inappropriate contacts with other molecules within their local environment. There is evidence that in some cases rather extensive nonnative interactions may form transiently to bury highly aggregation prone regions such as exposed hydrophobic surfaces (Hore *et al.*, 1997; Capaldi *et al.*, 2002). But to cope with this problem more generally, living systems have evolved a range of elaborate strategies to prevent interactions with other molecules prior to the completion of the folding process (Hartl and Hayer-Hartl, 2002).

One of the most important of the mechanisms to protect against aggregation is the large number of molecular chaperones that are present in all types of cells and cellular compartments. Despite their similar general role in enabling efficient folding and assembly, their specific functions can differ substantially, and it is evident that many types of chaperone work in tandem with each other (Ellis and Hartl, 1999; Hartl and Hayer-Hartl, 2002). Some molecular chaperones have been found to interact with nascent chains as they emerge from the ribosome, and bind rather nonspecifically to protect aggregation-prone regions rich in hydrophobic residues. Others are involved in assisting at later stages of the folding or assembly process. The most intensively studied molecular chaperone is the bacterial “chaperonin” GroEL and its cochaperone GroES, and many of the details of the mechanism through which this system functions are now well understood (Ellis, 1996; Hartl and Hayer-Hartl, 2002). A remarkable aspect of GroEL is that it contains a cavity in which polypeptide chains can be sequestered during folding and protected from the external environment. In addition to molecular chaperones, there are several types of folding catalyst that accelerate steps in the folding process that might otherwise be extremely slow (Hartl and Hayer-Hartl, 2002). The most important are peptidylprolyl isomerases, that increase the rate of *cis/trans* isomerization of peptide bonds involving proline residues, and protein disulphide isomerases that enhance the rate of formation and reorganization of disulphide bonds within proteins (Balbach and Schmid, 2000; Schiene and Fisher, 2000).

Because of the complexity of folding, misfolding is an inherent feature of the folding process for all proteins, particularly under adverse conditions. Misfolding can broadly be defined as reaching a state that has a significant proportion of nonnative interactions between residues and whose properties differ significantly from those of a similar state having overwhelmingly native-like interactions. The cellular levels of many chaperones are, for example, substantially increased during cellular stress, as their frequent designation as heat-shock proteins (Hsps) indicates (Pelham, 1986). Some molecular chaperones act to capture misfolded proteins, or even some types of aggregates, and provide them with another opportunity to fold correctly (Shorter and Lindquist, 2004). Such active intervention requires energy, and adenosine 5'-triphosphate (ATP) is required for many of the molecular chaperones to function correctly (Ellis and Hartl, 1999). Despite the fact that many molecular chaperones are usually at very high levels only in stressed systems, it is clear that they have a critical role to play in all organisms even when present at lower levels under normal physiological conditions. Moreover, in eukaryotic systems, many proteins that are synthesized in a cell are destined for secretion to an extracellular environment. These proteins are translocated into the ER where folding takes place prior to secretion through the Golgi apparatus. The ER contains a wide range of molecular chaperones and folding catalysts to promote efficient folding, and in addition, the proteins involved are subject to stringent "quality control" prior to secretion **Figure 2-2; see color insert** (Sitia and Braakman, 2003). Unfolded and misfolded proteins detected in this way are then targeted for degradation through the ubiquitin-proteasome pathway (Ellis and Hartl, 1999; Kaufman, 2002). The importance of the quality control process is underlined by the fact that recent experiments indicate that perhaps a third of all polypeptide chains may fail to satisfy the quality control mechanism in the ER, and for some proteins the success rate is even lower (Schubert *et al.*, 2000). Like the "heat-shock response" in the cytoplasm, the "unfolded protein response" in the ER is also upregulated during stress and, as we shall see below, is strongly linked to the avoidance of misfolding diseases.

Because of the importance of proteins in all biological processes, it is not surprising that failure to fold correctly, or to remain correctly folded, will give rise to the malfunctioning of living systems and therefore to disease. Indeed, it is increasingly evident that a large group of human diseases can be directly associated with aberrations in the folding process (Table 2-1) (Thomas *et al.*, 1995;

Table 2-1. Representative protein folding diseases

Disease	Protein	Site of folding
Hypercholesterolaemia	low-density lipoprotein receptor	ER
Cystic fibrosis	cystic fibrosis trans-membrane regulator	ER
Phenylketonuria	phenylalanine hydroxylase	cytosol
Huntington's disease	huntingtin	cytosol
Marfan syndrome	fibrillin	ER
Osteogenesis imperfecta	procollagen	ER
Sickle cell anaemia	hemoglobin	cytosol
α 1-antitrypsin deficiency	α 1-antitrypsin	ER
Tay-Sachs disease	β -hexosaminidase	ER
Scurvy	collagen	ER
Alzheimer's disease	β -amyloid/presenilin	ER
Parkinson's disease	α -synuclein	cytosol
Scrapie/Creutzfeldt-Jakob disease	prion protein	ER
Familial amyloidosis	transthyretin/lysozyme	ER
Retinitis pigmentosa	rhodopsin	ER
Cataracts	crystallins	cytosol
Cancer	p53	cytosol

From Dobson, 2001.

Dobson, 2001). Some of these diseases (e.g., cystic fibrosis) result from the fact that if proteins do not fold correctly they will not be able to exercise their proper function. In other cases, misfolded proteins escape all the protective mechanisms discussed above and form intractable deposits either within cells or in extracellular space. An increasing number of pathological conditions, including Alzheimer's and Parkinson's diseases, the spongiform encephalopathies and type II diabetes, are known to be directly associated with the deposition of specific proteins in a range of organs and tissues (Tan and Pepys, 1994; Pepys, 1995; Thomas *et al.*, 1995; Koo *et al.*, 1999; Dobson, 2001; Selkoe, 2003). As we shall see later, not only does this process result in a loss of function, but may be associated with a "toxic gain of function" in that the proteinaceous aggregates or their precursors can in some cases induce cell damage and cell death. Diseases associated with protein misfolding and aggregation are among the most debilitating, socially disruptive, and costly in the modern world, and they are becoming increasingly prevalent as our lifestyles change and our populations age (Dobson, 2002).

5. The Generic Nature of Amyloid Formation

One of the most characteristic features of many of the misfolding diseases is that they often give rise to the deposition of proteins in the form of amyloid fibrils and plaques (Tan and Pepys, 1994; Pepys, 1995; Thomas *et al.*, 1995; Koo *et al.*, 1999; Dobson, 2001; Selkoe, 2003). Such deposits can form in the brain, in other vital organs such as the liver and spleen, or in skeletal tissue, depending on the particular disorder. In the case of neurodegenerative diseases associated with aggregation, the quantities of the deposits can be very small, while in systemic diseases they may involve kilograms of protein. Each amyloid disease involves the aggregation of a specific protein, (Table 2-2) although a range of other components including other proteins and carbohydrates is also found to be associated with the deposits when they form *in vivo*. The soluble forms of the 20 or so proteins involved in the well-defined amyloidoses vary substantially—they range from large globular proteins to small and apparently unstructured peptides—but the aggregated forms have many common characteristics (Sunde and Blake, 1997). Amyloid deposits show specific optical properties, notably birefringence, on binding certain dye molecules such as Congo red; these properties have been used in post mortem diagnosis for more than 100 years. The fibrillar structures that are characteristic of many of the aggregates have very similar morphologies; they can be seen in electron or atomic force microscopy images to be long, unbranched, and often twisted structures, a few nanometers in diameter. Moreover, samples in which the fibrils can be at least partially aligned show a characteristic "cross- β " pattern in X-ray fibre diffraction experiments (Sunde and Blake, 1997). The latter indicates that the core structure of the fibrils is made up of β -sheets whose component strands run perpendicular to the fibril axis (**Figure 2-3; see color insert**) (Jiménez *et al.*, 2002). Fibrils having all the characteristics of *ex vivo* deposits can be reproduced *in vitro* from the component proteins under carefully chosen conditions, showing that they can self-assemble in the absence of any additional cellular components.

It was widely assumed until recently that the ability to form amyloid fibrils was limited to a relatively small number of proteins, largely those seen in disease states, and that these proteins must possess some specific sequence motifs encoding this apparently aberrant structure. Studies have now shown, however, that the ability of polypeptide chains to form such structures is common, and indeed may be considered a generic feature of polypeptide chains (Chiti *et al.*, 1999; Dobson, 1999). In particular, it has been shown that fibrils can be formed by many proteins that are not associated with disease once they are placed under conditions that destabilize the native structures, including such well-known proteins as myoglobin (Fändrich *et al.*, 2001; Stefani and Dobson, 2003), as Table 2-3 shows. The fact that specific sequences of amino acids are not essential for forming amyloid structures comes from the demonstration that homopolymers such as polythreonine or polylysine can also form

Table 2-2. Examples of diseases associated with amyloid deposition

Clinical syndrome	Fibril component	a: Organ limited b: Systemic
Alzheimer's disease	Aβ peptide, 1–40, 1–42	a
Spongiform encephalopathies	full-length prion protein or fragments	a
Primary systemic amyloidosis	intact light chain or fragments	b
Secondary systemic amyloidosis	76-residue fragment of amyloid A protein	b
Familial amyloidotic poly neuropathy I	transthyretin variants and fragments	b
Senile systemic amyloidosis	wild-type transthyretin and fragments	b
Hereditary cerebral amyloid angiopathy	fragment of cystatin-C	a
Hemodialysis-related amyloidosis	β2-microglobulin	b
Familial amyloidotic polyneuropathy II	fragments of apolipoprotein A-I	b
Finnish hereditary amyloidosis	71-residue fragment of gelsolin	b
Type II diabetes	islet-associated polypeptide (IAPP)	a
Medullary carcinoma of the thyroid	calcitonin	a
Atrial amyloidosis	atrial natriuretic factor	a
Lysozyme amyloidosis	full-length lysozyme variants	b
Insulin-related amyloidosis	full-length insulin	b
Fibrinogen α-chain amyloidosis	fibrinogen α-chain variants	b

Adapted from Dobson, 2001, and Selkoe, 2003.

Table 2-3. Representative proteins unrelated to disease that form amyloid fibrils *in vitro*

SH3 domain p85 phosphatidylinositol-3-Kinase (bovine)
Fibronectin type III module (murine)
Acylphosphatase (equine)
Monellin (<i>Dioscoreophyllum camminsii</i>)
Phosphoglycerate kinase (yeast)
Apolipoprotein CII (human)
ADA2H (human)
Met aminopeptidase (<i>Pyrococcus furiosus</i>)
Apo-cytochrome c (<i>Hydrogenobacter thermophilus</i>)
HypF N-terminal domain (<i>Escherichia coli</i>)
Apomyoglobin (equine)
Amphoterin (human)
Curlin CgsA subunit (<i>Escherichia coli</i>)
V1 domain (murine)
Fibroblast growth factor (<i>Notophthalmus viridescens</i>)
Stefin B (human)
Endostatin (human)

Adapted from Stefani and Dobson, 2003.

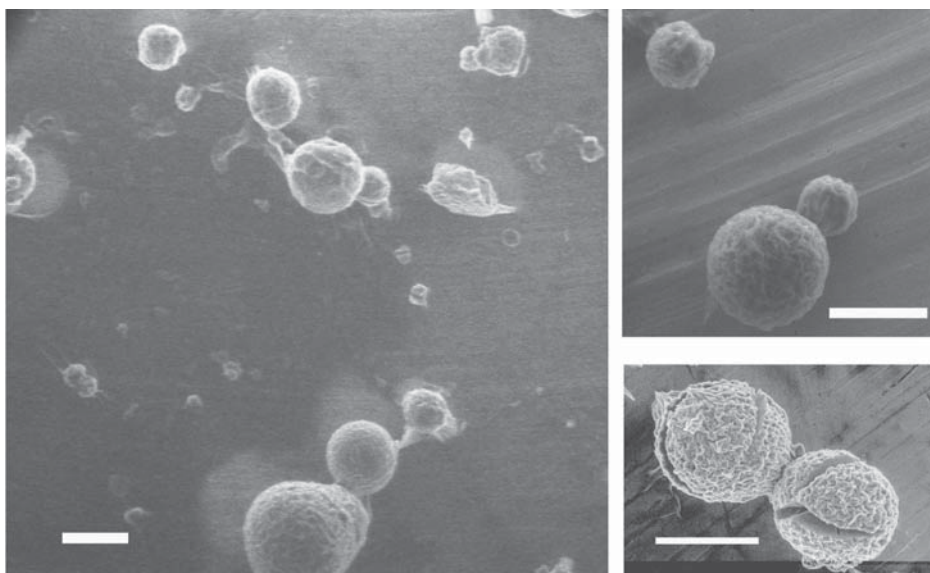


Figure 2-4. Images of insulin spherulites recorded by ESEM (environmental scanning electron microscopy). The scale bar is 50 μm . The picture at the bottom right illustrates some spherulites that have fractured. Optical experiments using crosspolarizers show that the structure involves a spherical lamellar array in which the amyloid fibrils are oriented tangentially to the lamellae (Krebs *et al.*, 2004).

very well-defined amyloid fibrils under appropriate conditions (Fändrich and Dobson, 2002). Moreover, fibrils of similar appearance to those containing large proteins can be formed by peptides with just a handful of amino acid residues (Lopez de al Puz *et al.*, 2002). The conditions under which amyloid structures form, rather than, for example, amorphous aggregates, vary with the characteristics of the sequence involved. A search for appropriate conditions, therefore, can involve a systematic screening exercise while varying parameters such as pH and temperature, in much the same way as a search is made for conditions under which the native states of proteins can be crystallized. One can consider that amyloid fibrils are the most highly organized structures adopted by unfolded polypeptide chains, and therefore will form most readily under conditions of relatively slow growth (Zurdo *et al.*, 2001). Indeed, the formation of amyloid fibrils can be considered to be the type of behavior that might be expected if polypeptide chains were to behave as simple polymer molecules. It is now evident, for example, that amyloid fibrils can form higher order assemblies such as spherulites (Figure 2-4), structures with diameters that are typically tens of microns and that have been known for synthetic polymers such as polyethylene for 50 years (Krebs *et al.*, 2004).

A great deal of effort has recently been directed at the determination of the detailed molecular structures of amyloid fibrils (Sunde and Blake, 1997; Jiménez *et al.*, 1999, 2002; Wille *et al.*, 1999; Serpell *et al.*, 2000; Petkova *et al.*, 2002; Jaroniec *et al.*, 2004). It is clear that the core structure of the fibrils involves interactions, particularly hydrogen bonds, between polypeptide chains that are largely extended. As only the main chain is common to all polypeptides, the side chains must be packed within the fibrils in whatever way is most favorable for a given sequence. Although the side chains do not determine the core structure, they do affect the details of the fibrillar assembly such

as their exact dimensions and the readiness with which they form (Chamberlain *et al.*, 2000). Moreover, in some cases it appears as if only a small proportion of the total polypeptide chain is incorporated in the core sheet structure, with the remainder of the chain associated in some other manner with the fibrillar assembly. In other cases, particularly small peptides, the whole of the molecule may be arranged in the repetitive β -sheet structure (Jaroniec *et al.*, 2004). The generic amyloid structure contrasts strongly with the thousands of different structures that are adopted by native globular proteins (Branden and Tooze, 1999). In these structures the packing of the side chains must be the dominant factor, rather than the intrinsic preferences of the main chain that are revealed in structures such as amyloid fibrils (Dobson, 1999, Fändrich *et al.*, 2001, Fändrich and Dobson, 2002). The strands and helices so familiar in the structures of native proteins are then the most stable structures that the main chain can adopt in the folds that are primarily defined by interactions between the side chains. Native structures are normally, however, only marginally stable; minor changes in the solution conditions, such as pH and temperature, the structures can unfold and may then, at least under some circumstances, reassemble in the form of amyloid fibrils.

One of the primary features of the “generic model” of amyloid formation is that the ability to form fibrils is common but the relative propensities to form fibrils vary substantially with the polypeptide sequence (DuBay *et al.*, 2004). There is considerable evidence supporting this assumption. Indeed, the mutation of single amino acids in a 100-residue protein can change the rate at which aggregation occurs from in its denatured state by an order of magnitude or more (Chiti *et al.*, 2003). Furthermore, the change in aggregation rate caused by such mutations can be correlated with the predicted changes in properties such as charge, secondary structure propensity, and hydrophobicity (Chiti *et al.*, 2003). This correlation has been found to hold for a wide range of different sequences, a finding that strongly endorses the generic model of aggregation and amyloid formation. Analysis of the aggregation rates of different proteins can also be rationalized using similar ideas, showing that natural proteins vary in their aggregation potentials by factors of 10^5 or more (**Figure 2-5; see color insert**) (DuBay *et al.*, 2004). Of particular interest is that the group of proteins that have been found to be partially or completely unfolded even under physiological conditions, usually known as natively unfolded proteins, have sequences that are predicted to have very low aggregation propensities (DuBay *et al.*, 2004; Uversky and Fink, 2004). It is well established that aggregation, like crystallization or indeed protein folding, is a nucleated process (Harper and Lansbury, 1997). Interestingly, it has been suggested that the residues involved in nucleation of the folding of a globular protein could be distinct from those that nucleate its aggregation into amyloid fibrils (Chiti *et al.*, 2002). Such a situation could result from the different nature of the partially folded species that initiate the two processes, and would provide an opportunity for evolutionary pressure to select sequences that favor folding over aggregation.

The ability to understand some features of the aggregation process that results in amyloid fibrils has prompted investigation of the mechanism by which they are assembled from the precursor species. In the native states of globular proteins the polypeptide main chain is usually buried within the folded structure and, in addition, a variety of more subtle structural features have evolved to inhibit aggregation (Richardson and Richardson, 2002). Conditions that favor formation of amyloid fibrils from such proteins are those that stimulate at least partial unfolding, for example low pH or elevated temperature (Kelly, 1998; Chiti *et al.*, 1999). Because of the dominance of the globular fold in preventing aggregation, mutations that destabilize the native state are commonly involved in familial forms of amyloid disease (Harper and Lansbury, 1997; Chiti *et al.*, 2003). Fragmentation of proteins, through proteolysis or other means, is another mechanism of promoting amyloid formation. Thus, some amyloid disorders, including Alzheimer's disease, result from the aggregation of fragments of larger precursor proteins that are unable to fold in the absence of the remainder of the protein structure (see Table 2-2). Because of the requirement for nucleation of aggregation, experiments *in vitro* indicate that the

formation of fibrils, by appropriately destabilized or fragmented proteins, is generally characterized by a lag phase, followed by a period of rapid growth. By analogy with crystallization, such a lag phase can be eliminated by addition of pre-formed fibrils to fresh solutions, a process known as seeding (Harper and Lansbury, 1997). Although the details of the events taken place during fibril growth are not yet known in detail, the overall kinetic profiles can often be simulated by using relatively simple models that incorporate well-established principles of nucleated processes (Padrick and Miranker, 2002; Chien *et al.*, 2004).

It appears from studies carried out so far that there are many common features in the mechanism of formation of amyloid fibrils by different peptides and proteins (**Figure 2-5; see color insert**) (Harper and Lansbury, 1997; Koo *et al.*, 1999; Nettleton *et al.*, 2000, Caughey and Lansbury, 2003). The first phase of the aggregation process involves the formation of oligomeric species as a result of relatively nonspecific interactions, although in some cases specific structural transitions such as domain swapping (Schlunegger *et al.*, 1997; Rousseau *et al.*, 2004) may be involved if such processes increase the rate of aggregation. At least the smaller oligomers are likely to be relatively soluble, and the earliest species visible by electron or atomic force microscopy as aggregation occurs generally resemble small bead-like structures, sometimes linked to one another, often described as amorphous aggregates or as micelles. These early “prefibrillar aggregates” then appear to transform into species with more distinctive morphologies, sometimes described as “protofibrils” or “protofilaments” (Jiménez *et al.*, 2002; Bitan *et al.*, 2003; Caughey and Lansbury, 2003). These latter structures are commonly short, thin, sometimes curly, fibrillar species that are thought, in some cases at least, to self-assemble into mature fibrils, perhaps by lateral association accompanied by some degree of structural reorganization as indicated in **Figure 2-6; see color insert** (Bouchard *et al.*, 2000; Plakoutsi *et al.*, 2004). The extent to which dissolution and reassembly of monomeric species is involved at the different stages of amyloid assembly is not clear, but such processes could well be important under the conditions of slow growth that frequently generate fibrils with the most regular appearance. The earliest aggregates are likely to be relatively disorganized structures that expose on their surfaces a variety of segments of the protein that are normally buried in the globular state. In other cases these early aggregates appear to be quite distinctive structures, including well-defined “doughnut”-shaped species seen for a number of systems (Lashuel *et al.*, 2002; Malisauskas *et al.*, 2003; Chien *et al.*, 2004). A particularly exciting development that promises to stimulate significant progress in studies of the mechanism of amyloid formation is the recent ability to use single molecule optical techniques to follow fibril growth in real time (Ban *et al.*, 2003).

6. Common Features of Protein Self-Assembly

Under particular conditions, a protein can adopt one or a number of more or less distinct states that can be represented schematically, as shown in **Figure 2-7; see color insert** (Dobson, 2003). The populations of the different states under a given set of conditions will depend on their relative thermodynamic stabilities and the rates at which they interconvert. Amyloid fibrils are just one of the types of aggregates that can be formed by a protein. They have particular significance, however, because their highly organized hydrogen-bonded structure is likely to give greater kinetic stability than more amorphous aggregates. This diagram emphasizes the importance for biological systems of controlling and regulating the various states accessible to a given polypeptide chain at given times and under given conditions. Indeed, such regulation is at least as important as the regulation and control of the various chemical transformations that take place in the cell (Dobson, 2003). The latter is achieved primarily through enzymes, and the former by means of the molecular chaperones and

the mechanisms for protein degradation, mentioned above. In a similar way that the aberrant behavior of enzymes can cause metabolic disease, the aberrant behavior of the chaperone and other machinery regulating polypeptide conformations can contribute to misfolding and aggregation diseases (Macario and De Macario, 2002).

The details of specific diseases associated with protein misfolding will be discussed in detail in later articles in this volume. The concepts represented in **Figure 2-7; see color insert**, however, serve as a general framework for understanding the molecular events that underlie such diseases, and indeed the principles that can be used to intervene for therapeutic purposes (Dobson, 2004a). As we have discussed above, it is partially or completely unfolded polypeptides that are particularly highly aggregation-prone. Such species will inevitably be generated during folding, and a variety of molecular chaperones is present in abundance in the cellular compartments wherever such processes occur. It is interesting in this regard that the majority of the deposits associated with aggregation diseases are extracellular; indeed, all the classical amyloidoses involve such aggregates, but in disorders such as Parkinson's and Huntington's diseases similar aggregates are found but here they are located within cells (Selkoe, 2003). It is therefore particularly important that proteins are correctly folded prior to their secretion from the cell; hence, the need for a highly effective system of quality control in the ER. Recently, however, extracellular chaperones have been identified, and these are likely to be of particular interest in the context of amyloid diseases (Wilson and Easterbrook-Smith, 2000). There is, of course, a continuous process of protein degradation in a normally functioning organism to eliminate misfolded as well as redundant proteins. During such processes, which require unfolding and proteolysis of polypeptide chains, aggregation may be a particularly danger. Degradation pathways, such as those of the ubiquitin–proteasome system, are therefore highly regulated to avoid such events (Bence *et al.*, 2001; Sherman and Goldberg, 2001).

In recent years our understanding of the detailed mechanism of all the processes associated with the complete life spans of proteins—from their synthesis to their degradation—has advanced dramatically through progress in both cellular and structural biology. High-resolution structures of ribosomes, proteasomes, molecular chaperones, and other complexes are revealing the details of how these complex molecular machines operate and are regulated. Structural techniques are also providing an increasing amount of information about the various states of the proteins that are processed by these assemblies. This task is particularly challenging as the majority of these species (such as unfolded and partially folded states and the less organized types of aggregates) are ensembles of more or less highly disordered structures, and in addition, may have only a transient existence. Major advances in the structural analysis of nonnative states of proteins are, however, now being made, particularly through the use of new approaches involving NMR spectroscopy (Petkova *et al.*, 2002; Vendruscolo and Dobson, 2003; Jaroniec *et al.*, 2004; Korzhnev *et al.*, 2004). It is therefore becoming possible to identify and characterize the various species that are represented in **Figure 2-7; see color insert** for specific proteins, and hence, to begin to understand the factors determining their behavior in different contexts (Dobson, 2003, 2004a).

To understand misfolding and aggregation diseases we need to know not just how such systems function under normal conditions, but also why they fail to function under other circumstances (Zurdo *et al.*, 2001; Horwich, 2002). The effects of many pathogenic mutations can be particularly well understood from the schematic representation given in **Figure 2-6; see color insert**. Many of the mutations associated with the familial deposition diseases increase the populations of partially unfolded states by decreasing the stability or cooperativity of the native state (Booth *et al.*, 1997; Ramirez-Alvarado *et al.*, 2000; Dumoulin *et al.*, 2003). Cooperativity is perhaps one of the most important characteristics of globular proteins that enables them to resist aggregation (Dobson and Karplus, 1999; Dobson, 1999, 2003). It ensures that even for proteins that are only marginally stable

in their native states, as the vast majority are, the population of unfolded molecules, or of unfolded regions of the polypeptide chain under physiological conditions, is minimal. Other familial amyloid diseases are associated with the accumulation of fragments of native proteins, produced by normal or aberrant processing or incomplete degradation; such species are unable to fold into aggregation-resistant states. Other pathogenic mutations act by enhancing the propensities of such species, or of natively unfolded proteins, to aggregate, for example by increasing their hydrophobicity or decreasing their charge (Chiti *et al.*, 2003). In the case of the transmissible encephalopathies, it is likely that ingestion of preaggregated states of an identical protein (e.g., through consumption of tissue from the same species or through contamination of surgical instruments or even perhaps by blood transfusion) increases dramatically the inherent rate of aggregation, and hence, underlies the mechanism of transmission (Harper and Lansbury, 1997; Prusiner, 1997; Chien *et al.*, 2003). Indeed, it has been shown recently that prion diseases can be induced in laboratory animals by injecting amyloid aggregates produced from purified recombinant prion protein, that no suggesting factors are necessary to generate disease (Legname *et al.*, 2004).

7. Generic Aspects of Misfolding Diseases

An extremely important question to be answered in the context of all the misfolding diseases is exactly how protein aggregation generates the clinical manifestations of the various conditions. In the case of systemic amyloid disease, the accumulation of large quantities of insoluble protein aggregates may itself disrupt the functioning of the organs in which they are located (Pepys, 1995). In other cases it may be that the loss of functional protein results in the failure of some crucial cellular process (Caughey and Lansbury, 2003). But for neurodegenerative disorders, such as Alzheimer's disease, it appears likely that the primary symptoms result from the destruction of neurons by mechanisms associated with the aggregation process (Koo *et al.*, 1999; Horwich, 2002; Caughey and Lansbury, 2003; Selkoe, 2003; Stefani and Dobson, 2003). It has recently been found that the early prefibrillar aggregates of proteins associated with neurological diseases can be highly damaging to cells; by contrast, the mature fibrils are often relatively benign (Walsh *et al.*, 2002; Selkoe, 2003). Investigation of the effects of aggregates on cells in culture indicates, however, that the toxic nature of protein aggregates is not restricted to species formed from the peptides and proteins associated with pathological conditions. Indeed, experiments have recently indicated that prefibrillar aggregates of proteins that are not connected with any known diseases can be as toxic to cells as those of the A β peptides (Bucciantini *et al.*, 2002).

It appears that the precursor aggregates of amyloid fibrils can readily pass through cell membranes and trigger a series of biochemical events that leads to cell death by apoptosis or necrosis (Bucciantini *et al.*, 2004). It may well be the case that small aggregates are toxic because they contain highly disordered polypeptide chains that display a large variety of amino acids, including those with hydrophobic side chains, on their surfaces resulting in aberrant interactions with a variety of cellular components including membranes (Polverino *et al.*, 2003; Stefani and Dobson, 2003; Bucciantini *et al.*, 2004). The generic nature of such aggregates and their effects on cells has recently been reinforced through experiments with polyclonal antibodies raised against small aggregates of A β peptides. These antibodies have been found to crossreact with early aggregates of different peptides and proteins and, moreover, to inhibit their toxicity in cell assays (Kayed *et al.*, 2003). That antibodies are able to recognize the common features of the early aggregates of different polypeptides may at first sight be surprising, particularly as these antibodies recognize neither the monomeric proteins nor the mature fibrils (Dumoulin and Dobson, 2005). But, as we have noted earlier, molecular chaperones appear to be able to recognize common features of misfolded proteins, such as segments of high hydrophobicity

(Hartl and Hayer-Hartl, 2002). Moreover, antibodies have been found that recognize mature fibrils formed by a range of different proteins (O'Nuallian and Wetzel, 2002). Moreover, *in vivo*, most amyloid deposits are associated with serum amyloid protein (SAP), a protein that is thought to act to inhibit the clearance of these deposits within the body; indeed, SAP labeled with radioactive isotopes is used to image amyloid deposits for clinical purposes (Pepys *et al.*, 2002).

Such findings raise the question as to how cellular systems are normally able to deal with the intrinsic tendency of incompletely folded proteins to aggregate. The answer is likely to be that the molecular chaperones and other "housekeeping" mechanism are remarkably efficient in ensuring that such potentially toxic species are destroyed or otherwise rendered harmless under normal circumstances (Bucciantini *et al.*, 2002; Hartl and Hayer-Hartl, 2002). Molecular chaperones of various types are able to shield hydrophobic regions of unfolded proteins, whether monomeric or present as small oligomers, to solubilize some forms of aggregates, or to alter the partitioning between different forms of aggregates (Hartl and Hayer-Hartl, 2002). The latter mechanism, for example, could, in addition to suppressing toxicity, prevent the precursors of amyloid fibrils converting into intractable species, thereby allowing them be refolded or disposed of by the cellular degradation systems. Indeed, evidence has been obtained that such a situation occurs with polyglutamine sequences associated with Huntington's disease where the precursor species appear to be diverted into amorphous aggregates by the combined action of two molecular chaperones, Hsp70 and Hsp40 (Muchowski *et al.*, 2000). If all such protective processes fail, it may still be possible for potentially harmful species to be sequestered in relatively harmless forms such as inclusion bodies in bacteria or aggresomes in eukaryotic systems. Indeed, it has been suggested that the formation of mature amyloid fibrils and plaques, whose toxicity appears to be much lower than that of their precursors as we have discussed above, may itself represent a protective mechanism under some circumstances (Koo *et al.*, 1999; Caughey and Lansbury, 2003; Stefani and Dobson, 2003).

Most of the aggregation diseases are, however, not associated with specific genetic mutations or infectious agents but with old age. The ideas summarized in this article offer a qualitative explanation of why this could be the case. We have seen that all proteins have an inherent tendency to aggregate unless they are maintained in a highly regulated and controlled environment. There is evidence from a variety of studies of protein sequences that selective pressure during evolution has resulted in avoidance of patterns of amino acid residues that are known to promote β -sheet formation and aggregation (Broome and Hecht, 2000). But just as random mutations tend to decrease protein stability, they are likely to enhance aggregation propensities, with the results that sequences evolve to be sufficiently stable or aggregation-resistant for their biological functions, but not to have such characteristics optimized further. We can see, therefore, that our recent ability to prolong life for a high proportion of the human population well beyond the normal reproductive age, that is, beyond the time that evolutionary pressure is significant, could itself lead to the proliferation of these diseases (Dobson, 1999, 2002). In old age it is likely that the probability of misfolded or aggregated species is increased and that the efficacy of the protective mechanisms is reduced (Csermely, 2001; Dobson, 2002; Macario and de Macario, 2002). It is intriguing, however, to speculate, that favorable mutations in aggregation-prone proteins might be the reason that some people do not readily succumb even in extreme old age to disorders such as Alzheimer's disease (Chiti *et al.*, 2003).

In addition to extended life spans, many other risk factors associated with sporadic forms of amyloid diseases are also associated with relatively recent changes of the life styles associated with human societies (Dobson, 2002). The transmissible prion diseases such as scrapie and bovine spongiform encephalopathy (BSE) are associated with intensive farming where the population density of animals is higher than in the wild, and particularly with modern practices of feeding animals with protein rendered from old animals of the same species. Chronic wasting disease, affecting large proportions of some of the deer and elk populations of North America, appears to be a very recent

phenomenon, perhaps associated with increasing numbers in the wild or the practice of deer farming (Miller and Williams, 2003). Similarly, the present dramatic increases in type II diabetes, estimated to affect up to 15% of the populations of some countries, is associated with obesity due to changing diets and reduced exercise (Höppener *et al.*, 2002). Some modern medical procedures have also been found to result in transmission of prion diseases, for example, Creutzfeldt-Jakob disease (CJD) cases have been shown to arise from treatment with contaminated growth hormone extracted from cadavers (Prusiner, 1997), or the amyloid deposition associated with hemodialysis, where the concentration of β -2 microglobulin in serum increases as the result of its failure to be cleared by the kidneys (Gejyo *et al.*, 1986). Indeed, it seems probable that in many countries of the world, health problems associated with amyloid disorders will soon be more costly, in both economic and social terms, than either heart disease or cancer.

8. Common Strategies for Therapeutic Intervention

Many of the amyloid diseases have no effective treatment at the present time, and those therapies that do exist can require radical intervention, for example, transplantation of organs such as the liver in some of the systemic amyloidoses (Stangou *et al.*, 1999). Some promising potential pharmaceuticals for specific amyloid diseases have arisen through serendipity or large-scale screening, but a better understanding of the underlying origins of amyloid disorders and their relatives gives hope that more rational approaches may soon bear fruit (Cohen and Kelly, 2003; Dobson, 2004a). The number of distinct steps in the process of converting a normally soluble polypeptide chain into toxic species or intractable deposits suggests that there is a variety of targets for possible therapeutic intervention. An important development in this regard is improved methodology for detecting amyloid deposits within living systems. Although the large-scale deposits of proteins associated with systemic diseases can be imaged, as described above, by use of radiolabeled SAP, the detection of aggregates involved in neurodegenerative conditions has proven to be particularly challenging. There have, however, been recent developments that show great promise, particularly involving analogues of the dye molecules that are widely used to monitor the formation of amyloid structures *in vitro* or their presence in tissue sections taken from patients during post mortem examination. By appropriate isotopic labeling of such compounds it has been possible to detect amyloid deposits in living subjects using positron emission tomography (Klunk *et al.*, 2004). Such developments should enable the efficacy of potential drugs to be monitored much more rapidly and quantitatively than in the past, at least if the drugs are designed to inhibit the aggregation process that leads directly to amyloid deposition.

The diagram shown in **Figure 2-8; see color insert** shows schematically the specific steps at which intervention could be envisaged on the basis of the ideas discussed earlier in this article (Dobson, 2004a). Indeed, it is reassuring that almost all of the current strategies for preventing or treating the amyloid disorders can be rationalized on such a picture. Step A, for example, involves the stabilization of the native state of an amyloidogenic protein to reduce its ability to expose aggregation-prone regions of the polypeptide chain to the outside world. Examples of this strategy include the use of analogues of the hormone thyroxine, the natural ligand for transthyretin, the protein associated with particularly prevalent types of amyloidoses (Hammarstrom *et al.*, 2003). Such ligands have been found to reduce the rate at which disease-associated mutational variants of this protein aggregate *in vitro*. Similarly, specific antibodies raised against wild-type lysozyme prevent the *in vitro* formation of amyloid fibrils by pathogenic forms of this protein (Dumoulin *et al.*, 2003; Dumoulin and Dobson, 2005). Of particular interest is quinacrine, an early antimalarial drug that was found to inhibit the replication of the pathogenic forms of the prion protein in cell cultures during screening

trials of approved pharmaceuticals known to cross the blood–brain barrier (May *et al.*, 2003). There is evidence that quinacrine acts to stabilize the soluble form of the prion protein, and clinical trials have been initiated to investigate its potential in treating CJD (Cohen and Kelly, 2003).

In the case of amyloid diseases that are associated with peptide fragments of larger proteins (see Table 2-3), a possible therapeutic approach is to reduce the levels of the aggregation-prone species by inhibiting the enzymes that generate them from their precursor protein, that is, by intervening at Step 2 of the schematic picture in **Figure 2-8; see color insert**. This approach has been investigated in particular for Alzheimer's disease where the 40 or 42 residue A β peptide forms the pathogenic aggregates. The strategy here is the development of inhibitors of the secretase enzymes that cleave the A β peptide from the membrane associated amyloid precursor protein (APP), and a number of promising candidates have been identified (Wolfe, 2002). Another approach in the future could be to use techniques such as gene therapy or stem cells to replace polypeptides whose aggregation is the origin of disease by mutational variants with lower aggregation propensities (Step C in **Figure 2-8; see color insert**). As we discussed above, studies of the kinetics of aggregate formation by a series of different peptides and proteins enables the prediction of mutations that are expected to decrease the rate of amyloid formation (Chiti *et al.*, 2003). This approach can be considered as a molecular analogue of liver transplantation for those familial amyloidoses where the therapeutic benefit arises because the mutant proteins associated with disease are primarily produced by this organ (Stangou *et al.*, 1999).

Another strategy to reduce the levels of aggregation-prone species in an organism is to increase the rate at which they are cleared (Step D in **Figure 2-8; see color insert**). One method of achieving this objective could be to generate antibodies to the polypeptides involved in the aggregation process. One particularly exciting approach has involved immunization with A β peptides to treat Alzheimer's disease (Schenk, 2002). Immunization has been shown to result in extensive clearance of amyloid deposits in a mouse model of the disease, and indeed, there is some evidence that similar effects occurred in human patients in initial clinical trials. Although these trials were terminated because of an inflammatory response in some of the patients, the results provide optimism that this therapeutic strategy has real potential either with improved vaccines or through passive immunotherapy involving infusion into patients of antibodies generated by external means. Another approach designed to induce enhanced clearance of amyloid deposits has involved the design of inhibitors of SAP, the protein discussed earlier in the context of the protection that it affords to amyloid deposits in the body (Pepys *et al.*, 2002). The more general principle of using rationally designed small molecules to target specific proteins for degradation is an exciting prospect.

An alternative strategy, represented as Step E in **Figure 2-8; see color insert**, involves prevention of the growth of fibrils by their amyloidogenic precursors rather than trying to decrease the levels of the latter within the body. A variety of peptides and peptide-analogues, often designated as β -breakers, have been designed to bind tightly to the ends of growing fibrils and to inhibit further growth. Again, the major target of such strategies has been Alzheimer's disease, and *in vitro* studies look very promising (Soto, 2003). Such species will need to be designed carefully so as not to generate increased concentrations of the oligomers and other fibril precursors that, as discussed above, appear to be the most damaging species in at least some of the neurodegenerative diseases. Last, but by no means least, is the possibility that the generic character of amyloid structures and the mechanism by which they are formed is sufficient that a strategy to address one of these diseases might be effective in preventing or treating others. At the very least one can be optimistic that the overall approach to developing effective therapeutics will be applicable to more than one of these diseases. More speculatively, it is at least possible that the same compounds could be successful in reducing the probability of becoming afflicted with any of the amyloid diseases. Compelling evidence that such a universal strategy based on the generic character of amyloid formation might be viable at least

in principle comes from the finding the antibodies raised against small aggregates of the A β peptides to recognize similar aggregates of other proteins (Kayed *et al.*, 2003).

9. Concluding Remarks

Proteins have evolved to fold efficiently and usually to remain correctly folded and soluble, despite the inherent tendency of polypeptide chains to aggregate. This remarkable achievement is undoubtedly a result of the natural selection of sequences and the coevolution of the environments in which they function. It is clear that as we develop further our knowledge of the mechanism of protein folding, and of the way that it is enhanced and regulated within the cellular environment, we shall be able to answer with increasing conviction the more general question of how evolution has enabled even the most complex biological systems to self-assemble with astonishing fidelity. Such knowledge will represent a very significant step towards understanding at a molecular level one of the most fascinating and fundamental characteristics of life itself (Vendruscolo *et al.*, 2003). Over the past century, however, we have begun to see the limitations of evolution in the context of the rapidity with which our environments and lifestyles are changing. Under such conditions one can see the inability of proteins to remain in their evolved and functional structures and their progressive conversion into the generic structures that reflect their fundamental polymeric nature. The mechanisms that have evolved to recognize, and render harmless, misfolded and aggregation-prone species are astonishingly efficient during the life spans of most individuals until the onset of old age (Dobson, 1999; Stefani and Dobson, 2003). This finding, along with evidence of common features in the manner that proteins convert into pathogenic species, suggest that it might be possible to enhance our natural defenses against aggregation in a variety of rational ways. If this possibility can be brought to fruition, it will provide an opportunity of improving significantly the quality of life in our aging societies, not only by preventing the suffering of those afflicted with these debilitating and disturbing disorders but also by reducing the burden of care that inevitably falls on the remainder of the population.

10. Abbreviations

A β	Amyloid β peptides
APP	Amyloid precursor protein
ATP	Adenosine 5'-triphosphate
BSE	Bovine spongiform encephalopathy
CJD	Creutzfeldt-Jakob disease
ER	Endoplasmic reticulum
Hsps	Heat-shock proteins
SAP	Serum amyloid protein

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References

- Balbach, J., and Schmid, F.X. (2000). Proline isomerization and its catalysis in protein folding. In: Pain, R.H. (Ed.), *Protein folding*, 2nd ed. Oxford: Oxford University Press, pp. 212–249.
- Ban, T., Hamada, D., Hasegawa, K., Naiki, H., and Goto, Y. (2003). Direct observation of amyloid fibril growth monitored by thioflavin T fluorescence. *J. Biol. Chem.* 278:16462–16465.
- Bence, N.F., Sampat, R.M., and Kopito, R.R. (2001). Impairment of the ubiquitin–proteasome system by protein aggregation. *Science* 292:1552–1555.
- Bitan, G., Kirkitadze, M.D., Lomakin, A., Vollers, S.S., Benedek, G.B., and Teplow, D.B. (2003). Amyloid beta-protein (A β) assembly: A β 40 and A β 42 oligomerize through distinct pathways. *Proc. Natl. Acad. Sci. USA* 100: 330–335.
- Booth, D.R., Sunde, M., Bellotti, V., Robinson, C.V., Hutchinson, W.L., Fraser, P.E., Hawkins, P.N., Dobson, C.M., Radford, S.E., Blake, C.C.F., and Pepys, M.B. (1997). Instability, unfolding and aggregation of human lysozyme variants underlying amyloid fibrillogenesis. *Nature* 385:787–793.
- Bouchard, M., Zurdo, J., Nettleton, E.J., Dobson, C.M., and Robinson, C.V. (2000). Formation of insulin amyloid fibrils followed by FTIR simultaneously with CD and electron microscopy. *Protein Sci.* 9:1960–1967.
- Branden, C., and Tooze, J. (1999). *Introduction to protein structure*. 2nd ed. New York: Garland Publishing.
- Broome, B.M., and Hecht, M.H. (2000). Nature disfavors sequences of alternating polar and non-polar amino acids: implications for amyloidogenesis. *J. Mol. Biol.* 296:961–968.
- Bucciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C.M., and Stefani, M. (2002). Inherent cytotoxicity of aggregates implies a common origin for protein misfolding diseases. *Nature* 416:507–511.
- Bucciantini, M., Calloni, G., Chiti, F., Formigli, L., Nosi, D., Dobson, C.M., and Stefani, M. (2004). Pre-fibrillar amyloid protein aggregates share common features of cytotoxicity. *J. Biol. Chem.* 279:31374–31382.
- Capaldi, A.P., Kleanthous, C., and Radford, S.E. (2002). Im7 folding mechanism: misfolding on a path to the native state. *Nat. Struct. Biol.* 9:209–216.
- Caughey, B., and Lansbury, P.T., Jr. (2003). Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders. *Annu. Rev. Neurosci.* 26:267–298.
- Chamberlain, A.K., MacPhee, C.E., Zurdo, J., Morozova-Roche, L.A., Hill, H.A., Dobson, C.M., and Davis, J.J. (2000). Ultrastructural organisation of amyloid fibrils by atomic force microscopy. *Biophys. J.* 79:3282–3293.
- Cheung, M.S., Garcia, A.E., and Onuchic, J.N. (2002). Protein folding mediated by solvation: water expulsion and formation of the hydrophobic core occur after the structural collapse. *Proc. Natl. Acad. Sci. USA* 99:685–690.
- Chien, P., DePace, A.H., Collins, S.R., and Weissman, J.S. (2003). Generation of prion transmission barriers by mutational control of amyloid conformations. *Nature* 424:948–951.
- Chien, P., Weismann, J.S., and DePace, A.H. (2004). Emerging principles of conformation-based prion inheritance. *Annu. Rev. Biochem.* 73:617–656.
- Chiti, F., Webster, P., Taddei, N., Clark, A., Stefani, M., Ramponi, G., and Dobson, C.M. (1999). Designing conditions for *in vitro* formation of amyloid protofilaments and fibrils. *Proc. Natl. Acad. Sci. USA* 96:3590–3594.
- Chiti, F., Taddei, N., Baroni, F., Capanni, C., Stefani, M., Ramponi, G., and Dobson, C.M. (2002). Kinetic partitioning of protein folding and aggregation. *Nat. Struct. Biol.* 9:137–143.
- Chiti, F., Stefani, M., Taddei, N., Ramponi, G., and Dobson, C.M. (2003). Rationalisation of mutational effects on peptide and protein aggregation rates. *Nature* 424:805–808.
- Cohen, F.E., and Kelly, J.W. (2003). Therapeutic approaches to protein-misfolding diseases. *Nature* 426:905–909.
- Csermely, P. (2001). Chaperone overload is a possible contributor to “civilization diseases.” *Trends Gen.* 17:701–704.
- Davis, R., Dobson, C.M., and Vendruscolo, M. (2002). Determination of the structures of distinct transition state ensembles for a β -sheet peptide with parallel folding pathways. *J. Chem. Phys.* 117:9510–9517.
- Debe, D.A., Carlson, M.J., and Goddard, W.A., III. (1999). The topomer-sampling model of protein folding. *Proc. Natl. Acad. Sci. USA* 96:2596–2601.
- Dill, K.A., and Chan, H.S. (1997). From Levinthal to pathways to funnels. *Nat. Struct. Biol.* 4:10–19.
- Dobson, C.M. (1999). Protein misfolding, evolution and disease. *Trends Biochem. Sci.* 24:329–332.
- Dobson, C.M. (2001). The structural basis of protein folding and its links with human disease. *Philos. Trans. R. Soc. Lond. B* 356:133–145.
- Dobson, C.M. (2002). Getting out of shape—protein misfolding diseases. *Nature* 418:729–730.
- Dobson, C.M. (2003). Protein folding and misfolding. *Nature* 426:884–890.

- Dobson, C.M. (2004a). In the footsteps of alchemists. *Science* 295:1719–1722.
- Dobson, C.M. (2004b). Principles of protein folding, misfolding and aggregation. *Semin. Cell Dev. Biol.* 15:3–16.
- Dobson, C.M., and Karplus, M. (1999). The fundamentals of protein folding: bringing together theory and experiment. *Curr. Opin. Struct. Biol.* 9:92–101.
- Dobson, C.M., Sali, A., and Karplus, M. (1998). Protein folding: a perspective from theory and experiment. *Angew. Chem. Int. Ed. Eng.* 37:868–893.
- DuBay, K.F., Pawar, A.P., Chiti, F., Zurdo, J., Dobson, C.M., and Vendruscolo, M. (2004). Prediction of the absolute aggregation rates of amyloidogenic polypeptide chains. *J. Mol. Biol.* 341:1317–1326.
- Dumoulin, M., and Dobson, C.M. (2005). Probing the origins, diagnosis and treatment of amyloid disease using antibodies. *Biochimie.* 86:589–600.
- Dumoulin, M., Last, A.M., Desmyter, A., Decanniere, K., Canet, D., Spencer, A., Archer, D.B., Muyldermans, S., Wyns, L., Matagne, A., Redfield, C., Robinson, C.V., and Dobson, C.M. (2003). A camelid antibody fragment inhibits amyloid fibril formation by human lysozyme. *Nature* 424:783–788.
- Ellis, R.J. (Ed.). (1996). *The chaperonins*. San Diego, CA: Academic Press.
- Ellis, R.J., and Hartl, F.U. (1999). Principles of protein folding in the cellular environment. *Curr. Opin. Struct. Biol.* 9:102–110.
- Ellis, R.J., and Minton, A.P. (2003). Join the crowd. *Nature* 425:27–28.
- Fändrich, M., and Dobson, C.M. (2002). The behaviour of polyamino acids reveals an inverse side-chain effect in amyloid structure formation. *EMBO J.* 21:5682–5690.
- Fändrich, M., Fletcher, M.A., and Dobson, C.M. (2001). Amyloid fibrils from muscle myoglobin. *Nature* 410:165–166.
- Fersht, A.R. (1999). *Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding*. New York: W.H. Freeman.
- Fersht, A.R. (2000). Transition-state structure as a unifying basis in protein-folding mechanisms: contact order, chain topology, stability, and the extended nucleus mechanism. *Proc. Natl. Acad. Sci. USA* 97:1525–1529.
- Fersht, A.R. and Daggett, V. (2002). Protein folding and unfolding at atomic resolution. *Cell* 108:573–582.
- Gejyo, F., Homma, N., Suzuki, Y., and Arakawa, M. (1986). Serum levels of β 2-microglobulin as a new form of amyloid protein in patients undergoing long-term hemodialysis. *N. Engl. J. Med.* 314:585–586.
- Gilbert, R.J.C., Fucini, P., Connell, S., Fuller, S.D., Nierhaus, K.H., Robinson, C.V., Dobson, C.M., and Stuart, D.I. (2004). Three-dimensional structures of translating ribosomes by cryo-EM. *Mol. Cell.* 14:57–66.
- Hammarstrom, P., Wiseman, R.L., Powers, E.T., and Kelly, J.W. (2003). Prevention of transthyretin amyloid disease by changing protein misfolding energetics. *Science* 299:713–716.
- Hardesty, B., and Kramer, G. (2001). Folding of a nascent peptide on the ribosome. *Prog. Nucleic Acid Res. Mol. Biol.* 66:41–66.
- Harper, J.D., and Lansbury, P.T., Jr. (1997). Models of amyloid seeding in Alzheimer's disease and scrapie: mechanistic truths and physiological consequences of the time-dependent solubility of amyloid proteins. *Annu. Rev. Biochem.* 66:385–407.
- Hartl, F.U., and Hayer-Hartl, M. (2002). Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* 295:1852–1858.
- Höppener, J.W.M., Nieuwenhuis, M.G., Vroom, T.M., Ahrén, B., and Lips, C.J.M. (2002). Role of islet amyloid in type 2 diabetes mellitus: consequence or cause? *Mol. Cell. Endocrinol.* 197:205–212.
- Hore, P.J., Winder, S.L., Roberts, C.H., and Dobson, C.M. (1997). Stopped-flow photo-CIDNP observation of protein folding. *J. Am. Chem. Soc.* 119:5049–5050.
- Horwich, A. (2002). Protein aggregation in disease: a role for folding intermediates forming specific multimeric interactions. *J. Clin. Invest.* 110:1221–1232.
- Jackson, S.E. (1998). How do small single-domain proteins fold? *Fold. Des.* 3:R81–R91.
- Jaroniec, C.P., MacPhee, C.E., Bajaj, V.S., McMahon, M.T., Dobson, C.M., and Griffin, R.G. (2004). High resolution molecular structure of a peptide in an amyloid fibril determined by magic angle spinning NMR spectroscopy. *Proc. Natl. Acad. Sci. USA* 101:711–716.
- Jenni, S., and Ban, N. (2003). The chemistry of protein synthesis and voyage through the ribosomal tunnel. *Curr. Opin. Struct. Biol.* 13:212–219.
- Jiménez, J.L., Gujjarro, J.I., Orlova, E., Zurdo, J., Dobson, C.M., Sunde, M. and Saibil, H.R. (1999). Cryo-electron microscopy structure of an SH3 amyloid fibril and model of the molecular packing. *EMBO J.* 18:815–821.
- Jiménez, J.L., Nettleton, E.J., Bouchard, M., Robinson, C.V., Dobson, C.M., and Saibil, H.R. (2002). The protofilament structure of insulin amyloid fibrils. *Proc. Natl. Acad. Sci. USA* 99:9196–9201.

- Karplus, M. (1997). The Levinthal paradox, yesterday and today. *Fold. Des.* 2:569–576.
- Karplus, M., and Weaver, D.L. (1994). Folding dynamics: the diffusion-collision model and experimental data. *Protein Sci.* 3:650.
- Kaufman, R.J. (2002). The unfolded protein response in nutrient sensing and differentiation. *Nat. Rev. Mol. Cell Biol.* 3:411–421.
- Kayed, R., Head, E., Thompson, J.L., McIntire, T.M., Milton, S.C., Cotman, C.W., and Glabe, C.G. (2003). Common structure of soluble amyloid oligomers implies common mechanisms of pathogenesis. *Science* 300:486–489.
- Kelly, J. (1998). Alternative conformation of amyloidogenic proteins and their multi-step assembly pathways. *Curr. Opin. Struct. Biol.* 8:101–106.
- Klunk, W.E., Engler, H., Nordberg, A., Wang, Y., Blomqvist, G., et al. (2004). Imaging brain amyloid in Alzheimer's disease with Pittsburgh Compound-B. *Ann. Neurol.* 55:306–319.
- Koo, E.H., Lansbury, P.T., Jr., and Kelly, J.W. (1999). Amyloid diseases: abnormal protein aggregation in neurodegeneration. *Proc. Natl. Acad. Sci. USA* 96:9989–9990.
- Korzhev, D.M., Salvatella, X., Vendruscolo, M., Di Nardo, A.A., Davidson, A.R., Dobson, C.M., and Kay, L.E. (2004). Low populated folding intermediates of the Fyn SH3 domain characterized by relaxation dispersion NMR. *Nature* 430:586–590.
- Krebs, M.R.H., MacPhee, C.E., Miller, A.F., Dunlop, I.E., Dobson, C.M., and Donald, A.M. (2004). The formation of spherulites by amyloid fibrils of bovine insulin. *Proc. Natl. Acad. Sci. USA* 101:14420–14424.
- Lashuel, H.A., Hartley, D., Petre, B.M., Walz, T., Lansbury, P.T., Jr. (2002). Neurodegenerative disease: amyloid pores from pathogenic mutations. *Nature* 418:291.
- Legname, G., Baskakov, I.V., Nguyen, H.O., Riesner, D., Cohen, F.E., DeArmond, S.J., and Prusiner, S.J. (2004). Synthetic mammalian prions. *Science* 305:673–676.
- Lindorff-Larsen, K., Vendruscolo, M., Paci, E., and Dobson, C.M. (2004). Transition states for protein folding have native topologies despite high structural variability. *Nat. Struct. Mol. Biol.* 11:443–439.
- Lopez de la Paz, M., Goldie, K., Zurdo, J., Lacrois, E., Dobson, C.M., Hoenger, A., and Serrano, L. (2002). *De novo* designed peptide-based amyloid fibrils. *Proc. Natl. Acad. Sci. USA* 99:16052–16057.
- Macario, A.J.L., and de Macario, E.C. (2002). Sick chaperones and ageing: a perspective. *Ageing Res. Rev.* 1:295–311.
- Makarov, D.E., and Plaxco, K.W. (2003). The topomer search model: a simple, quantitative theory of two-state protein folding kinetics. *Protein Sci.* 12:17–26.
- Malisauskas, M., Zamotin, V., Jass, J., Noppe, W., Dobson, C.M., and Morozova-Roche, L.A. (2003). Amyloid protofibrils from the calcium-binding protein equine lysozyme: formation of ring and linear structures depends on pH and metal ion concentration. *J. Mol. Biol.* 330:879–890.
- Matouschek, A., Kellis, J.T., Jr., Serrano, L., and Fersht, A.R. 1989. Mapping the transition state and pathway of protein folding by protein engineering. *Nature* 342:122.
- May, B.C., Fafarman, A.T., Hong, S.B., Rogers, M., Deady, L.W., Prusiner, S.B., and Cohen, F.E. (2003). Potent inhibition of scrapie prion replication in cultured cells by bisacridines. *Proc. Natl. Acad. Sci. USA* 100:3416–3421.
- Miller, M.W., and Williams, E.S. (2003). Horizontal prion transmission in male deer. *Nature* 425:35–36.
- Muchowski, P.J., Schaffar, G., Sittler, A., Wanker, E.E., Hayer-Hartl, M.K., and Hartl, F.U. (2000). Hsp70 and Hsp40 chaperones can inhibit self-assembly of polyglutamine proteins into amyloid-like fibrils. *Proc. Natl. Acad. Sci. USA* 97:7841–7846.
- Nettleton, E.J., Tito, P., Sunde, M., Bouchard, M., Dobson, C.M., and Robinson, C.V. (2000). Characterization of the oligomeric states of insulin in self-assembly and amyloid fibril formation by mass spectrometry. *Biophys. J.* 79: 1053–1065.
- O'Neill, B., and Wetzel, B. (2002). Conformational Abs recognizing a generic amyloid fibril epitope. *Proc. Natl. Acad. Sci. USA* 99:1485–1490.
- Paci, E., Friel, C., Lindorff-Larsen, K., Radford, S., Karplus, M., and Vendruscolo, M. (2004). Comparison of the transition states ensembles for folding of Im7 and Im9 determined using all-atom molecular dynamics simulations with Φ value restraints. *Proteins* 54:513–525.
- Padrick, S.B., and Miranker, A.D. (2002). Islet amyloid phase partitioning and secondary nucleation are central to the mechanism of fibrillogenesis. *Biochemistry* 41:4694–4703.
- Panchenko, A.R., Luthey-Schulter, Z., and Wolynes, P.G. (1996). Foldons, protein structural modules, and exons. *Proc. Natl. Acad. Sci. USA* 93:2008–2013.
- Pelham, H.R. 1986. Speculations on the functions of the major heat shock and glucose-regulated proteins. *Cell* 46: 959–961.

- Pepys, M.B. (1995). Amyloidosis. In: Weatherall, D.J., Ledingham, J.G.G., and Warrell, D.A. (Eds.). *The Oxford textbook of medicine*, 3rd ed. Oxford: Oxford University Press, pp.1512–1524.
- Pepys, M.B., et al. (2002). Targeted pharmacological depletion of serum amyloid P component for treatment of human amyloidosis. *Nature* 417:254–259.
- Petkova, A.T., Ishii, Y., Balbach, J.J., Antzutkin, O.N., Leapman, R.D., Delaglio, F., and Tycko, R. (2002). A structural model for Alzheimer's β -amyloid fibrils based on experimental constraints from solid state NMR. *Proc. Natl. Acad. Sci. USA* 99:16742–16747.
- Plakoutsi, G., Taddei, N., Stefani, M., and Chiti, F. (2004). Aggregation of the acylphosphatase from *Sulfolobus solfataricus*: the folded and partially unfolded states can both be precursors for amyloid formation. *J. Biol. Chem.* 279:14111–14119.
- Polverino de Lauro, P., Taddei, N., Frare, E., Capanni, C., Constantini, S., Zurdo, J., Chiti, F., Dobson, C.M., and Fontana, A. (2003). Protein aggregatin and amyloid fibril formation by an SH3 domain probed by limited proteolysis. *J. Mol. Biol.* 334:129–141.
- Prusiner, S. (1997). Prion diseases and the BSE crisis. *Science* 278:245–251.
- Ramirez-Alvarado, M., Merkel, J.S., and Regan, L. (2000). A systematic exploration of the influence of the protein stability on amyloid fibril formation *in vitro*. *Proc. Natl. Acad. Sci. USA* 97:8979–8984.
- Richardson, J.S., and Richardson, D.C. (2002). Natural β -sheet proteins use negative design to avoid edge-to-edge aggregation. *Proc. Natl. Acad. Sci. USA* 99:2754–2759.
- Rousseau, F., Schymkowitz, J.W., Wilkinson, H.R., and Itzhaki, L.S. (2004). Intermediates control domain swapping during folding of p13suc1. *J. Biol. Chem.* 279:8368–8377.
- Schenck, D. (2002). Amyloid- β immunotherapy for Alzheimer's disease: the end of the beginning. *Nat. Rev. Neurosci.* 3:824–828.
- Schiene, C., and Fisher, G. (2000). Enzymes that catalyse the restructuring of proteins. *Curr. Opin. Struct. Biol.* 10:40.
- Schlunegger, M.P., Bennett, M.J., and Eisenberg, D. (1997). Oligomer formation by 3D domain swapping: a model for protein assembly and misassembly. *Adv. Protein Chem.* 50:61–122.
- Schubert, U., Anton, L.C., Gibbs, J., Orbyry, C.C., Yewdell, J.W., and Bennink, J.R. (2000). Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* 404:770–774.
- Selkoe, D.J. (2003). Folding proteins in fatal ways. *Nature* 426:900–904.
- Serpell, L.C., Blake, C.C.F., and Fraser, P.E. (2000). Molecular structure of a fibrillar Alzheimer's A β fragment. *Biochemistry* 39:13269–13275.
- Sherman, M.Y., and Goldberg, A.L. (2001). Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. *Neuron* 29:15–32.
- Shorter, J., and Lindquist, S. (2004). Hsp104 catalyzes formation and elimination of self-replicating Sup35 pion conformers. *Science* 304:1793–1797.
- Sitia, R., and Braakman, I. (2003). Quality control in the endoplasmic reticulum folding factory. *Nature* 426:891–894.
- Soto, C. (2003). Unfolding the role of protein misfolding in neurodegenerative diseases. *Nat. Rev. Neurosci.* 4:49–60.
- Stangou, A.J., Hawkins, P.N., Booth, D.R., O'Grady, J., Jewitt, D., Rela, M., Pepys, M.B., and Heaton, N.D. (1999). Liver transplantation for non-Met30 TTR associated familial amyloid polyneuropathy. *Hepatology* 30:576.
- Stefani, M., and Dobson, C.M. (2003). Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. *J. Mol. Med.* 81:678–699.
- Sunde, M., and Blake, C.C. (1997). F, the structure of amyloid fibrils by electron microscopy and X-ray diffraction. *Adv. Protein Chem.* 50:123–159.
- Tan, S.Y., and Pepys, M.B. (1994). Amyloidosis. *Histopathology* 25:403–414.
- Thomas, P.J., Qu, B.H., and Pedersen, P.L. (1995). Defective protein folding as a basis of human disease. *Trends Biochem. Sci.* 20:456–459.
- Trieber, D.K., and Williamson, J.R. (1999). Exploring the kinetic traps in RNA folding, *Curr. Opin. Struct. Biol.* 9:339–345.
- Uversky, V.N., and Fink, A.L. (2004). Conformational constraints for amyloid fibrillation: the importance of being unfolded. *Biochim. Biophys. Acta* 1698:131–153.
- Vendruscolo, M., and Dobson, C.M. (2005). Towards complete descriptions of the free energy landscapes of proteins. *Philos. Trans. R. Soc. Lond. A* 363:433–452.
- Vendruscolo, M., Paci, E., Dobson, C.M., and Karplus, M. (2001). Three key residues form a critical contact network in a transition state for protein folding. *Nature* 409:641–646.
- Vendruscolo, M., Zurdo, J., MacPhee, C.E., and Dobson, C.M. (2003). Protein folding and misfolding: a paradigm of self-assembly and regulation in complex biological systems. *Philos. Trans. R. Soc. Lond. A* 361:1205–1222.

- Walsh, D.M., Klyubin, I., Fadeeva, J.V., Cullen, W.K., Anwyl, R., Wolfe, M.S., Rowan, M.J., and Selkoe, D.J. (2002). Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation *in vivo*. *Nature* 416:535–539.
- Wille, H., Michelitsch, M.D., Guenebaut, V., Supattapone, S., Serban, A., Cohen, F.E., Agard, D.A., and Prusiner, S.B. (1999). Structural studies of the scrapie prion protein by electron crystallography. *Proc. Natl. Acad. Sci. USA* 99: 3563–3568.
- Wilson, M.R., and Easterbrook-Smith, S.B. (2000). Clusterin is a secreted mammalian chaperone. *Trends Biochem. Sci.* 25:95–98.
- Wolfe, M.S. (2002). Secretase as a target for Alzheimer's disease. *Curr. Top. Med. Chem.* 2:371–383.
- Wolynes, P.G., Onuchic, J.N., and Thirumalai, D. (1995). Navigating the folding routes. *Science* 267:1619–1623.
- Woolhead, C.A., McCormick, P.J., and Johnson, A.E. (2004). Nascent membrane and secretory proteins differ in FRET-detected folding far inside the ribosome and in their exposure to ribosomal proteins. *Cell* 116:725–736.
- Yusupov, M.M., Yusupova, G.Z., Baucom, A., Lieberman, K., Earnest, T.N., Cate, J.H., and Noller, H.F. (2001). Crystal structure of the ribosome at 5.5 Å resolution. *Science* 292:883–896.
- Zurdo, J., Guijarro, J.I., Jiménez, J.L., Saibil, H.R., and Dobson, C.M. (2001). Dependence on solution conditions of aggregation and amyloid formation by an SH3 domain. *J. Mol. Biol.* 311:325–340.

Relative Importance of Hydrophobicity, Net Charge, and Secondary Structure Propensities in Protein Aggregation

Fabrizio Chiti

1. Abstract

A full understanding of the mechanism by which proteins and peptides convert from their soluble states into amyloid aggregates requires a detailed elucidation of the sequence and structural determinants that govern the processes of amyloid formation. The experimental results collected in the past few years converge on the idea that hydrophobicity, propensity to form a secondary structure and charge are key determinants of aggregation. The effect of mutations on aggregation and the reasons why particular regions of the sequence are more effective than others in promoting aggregation of unstructured polypeptide chains can be explained on the basis of these physicochemical factors. At present, it is possible to edit algorithms to calculate the relative effects of mutations on aggregation, the absolute aggregation rate of a natively unfolded protein, and to identify regions of the sequence that play key roles in promoting the aggregation process of the whole protein. In addition, because amyloid formation seems to be a shared property of natural proteins, protein sequences appear to have evolved to reduce their propensities to aggregate. One of the strategies by which proteins have achieved this end has been to modulate their aggregation potential by playing with these factors.

2. Introduction

From the previous chapter we learned that the formation of highly organized amyloid aggregates is a generic property of polypeptides, and not simply a feature of the few proteins associated with recognized pathological conditions. In this chapter, I will focus on the major determinants that promote the conversion of unfolded or partially folded states into ordered aggregates. This process is of paramount importance for a number of reasons. First, many proteins or peptides that aggregate under pathological conditions are largely unstructured without well-defined three-dimensional structures. Examples include the amyloid β (A β) peptide associated with Alzheimer's disease and cerebral amyloid angiopathy, α -synuclein associated with Parkinson's disease and dementia with Lewy bodies, the τ protein associated with frontotemporal dementia with Parkinsonism, and the ABri and ADan peptides associated with British and Danish dementia, respectively (Selkoe, 2003; Stefani and Dobson, 2003; Uversky and Fink, 2004). Several proteins or protein fragments associated with non-neuropathic amyloidoses are also predominantly unstructured (Uversky and Fink, 2004), such as the

islet amyloid polypeptide associated with type II diabetes (Kayed *et al.*, 1999), calcitonin associated with medullary carcinoma of the thyroid (Thunecke and Fischer, 1998), and the protein fragment associated with hereditary apolipoprotein A1 amyloidosis (Mangione *et al.*, 2001). It is clear, therefore, that it is of very considerable medical importance to understand the determinants of aggregation propensities from unstructured states.

However, an understanding of aggregation from denatured states of proteins is not important only for relatively unstructured systems. It is a well-diffused opinion that amyloid fibrils from proteins normally adopting a globular and well-defined folded conformation start to aggregate after at least partial unfolding caused by mutation, change of conditions, or proteolysis (**Figure 3-1; see color insert**) (Lai *et al.*, 1996; Booth *et al.*, 1997; Kelly, 1998; McParland *et al.*, 2000; Rochet and Lansbury, 2000; Staniforth *et al.*, 2001; Dobson, 2003; Selkoe, 2003; reviewed by Uversky and Fink, 2004). Although this view is now challenged by recent observations that some proteins do not necessarily need to unfold substantially in order to form or initiate formation of well-ordered fibrillar aggregates (Bousset *et al.*, 2002; Laurine *et al.*, 2003; Plakoutsi *et al.*, 2004), it is still universally recognized that unfolded or partially unfolded states generally possess a considerably higher propensity to aggregate than fully native states. Aggregation from denatured states remains a fundamental problem in the biology of all folded proteins, because these systems can adopt nonnative conformations during or immediately after biosynthesis, under stress conditions, or as a consequence of mutation or proteolysis.

3. Importance of Hydrophobicity in Protein Aggregation

3.1. Analysis of Four Representative Cases

Hydrophobic interactions are particularly important driving forces in the aggregation processes of polypeptide chains. Several studies have shown that regions of the sequence of a protein that promote aggregation are particularly rich with hydrophobic residues. This paragraph will be focused on four protein sequences for which a large body of experimental data is available. These include the amyloid β peptide ($A\beta$), α -synuclein (α -syn), a fragment from τ (PHF43), and muscle acylphosphatase (AcP).

3.1.1. $A\beta$

Proline scanning mutagenesis and studies based on the dissection of the entire peptide into smaller fragments have indicated that the region of the sequence spanning approximately residues 15–21 is a key stretch in the process of amyloid formation (Tjernberg *et al.*, 1999; Williams *et al.*, 2004). This region also appears to form part of the β -structured core of the fibrils as determined by solid-state NMR and site-directed spin labeling (Petkova *et al.*, 2002; Torok *et al.*, 2002). The region immediately before the C-terminus and spanning residues 31–37 has been proposed to be another relevant portion of $A\beta$, as substitutions of residues within this region by proline produces considerable decrease in aggregation propensity (Williams *et al.*, 2004). Although the involvement of the last two to four residues in the cross- β structure of the fibrils is still debated, there is a general agreement that the 31–37 region is part of the β -core of the fibrillar aggregates (Petkova *et al.*, 2002; Torok *et al.*, 2002; Williams *et al.*, 2004). The 15–21 and 31–37 regions correspond to peaks of hydrophobicity in the hydropathy profile of the peptide (Figure 3-2A). It is therefore likely that aggregation of $A\beta$ is promoted by such hydrophobic portions of the polypeptide chain that will be subsequently structured in the resulting fibrils.

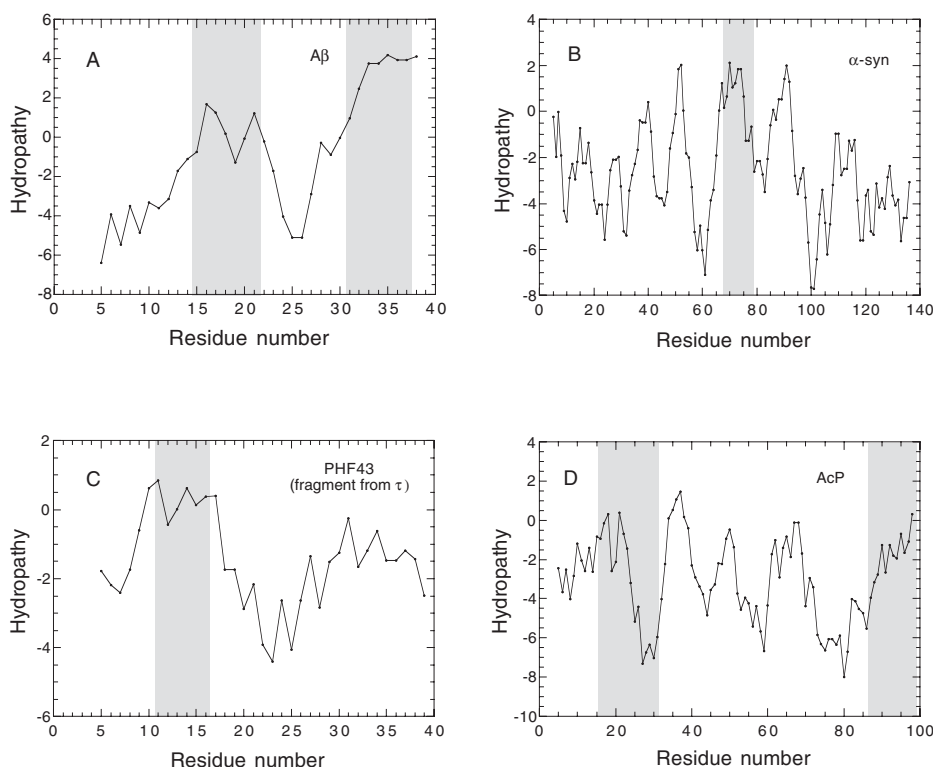


Figure 3-2. Hydropathy profiles of $A\beta_{1-42}$ (A), α -syn (B), the τ fragment PHF43 (C), and AcP (D). Hydropathy profiles were obtained using the protowork software package (Open-Lab, Florence, Italy). In brief, the software calculates the average hydrophobicity of windows of nine residues and assigns the calculated values to the central residue in each case. Given this procedure the profile starts at the residue no. 5 and ends at the N-4 residue (where N is the last residue). The hydrophobicity values used for the 20 amino acids are those reported by Roseman (1988), also published by Creighton (1993). Gray areas indicate regions of the sequence found experimentally to promote aggregation. In all cases such regions correspond to relatively high hydrophobic regions. See text for more details.

3.1.2. α -Syn

A large body of evidence indicates that the central portion of α -syn, generally referred to as NAC, and comprising residues 61–95, is the most relevant determinant of aggregation and fibrillization of this protein (reviewed in Uversky and Fink, 2002). Protein dissection studies have shown that the peptide 68–78 is the minimal NAC fragment retaining the ability to fibrillate (Bodles *et al.*, 2001). NAC peptides spanning residues external to this region do not fibrillate (El-Agnaf *et al.*, 1998; Bodles *et al.*, 2001). Similarly to $A\beta$, this region of the sequence embodies one of the major and most extended peak of hydrophobicity in the sequence of the entire protein (Figure 3-2B). Other investigators have shown that deletion of the 71–82 region in α -syn suppresses its fibrillization, while a short peptide encompassing these residues readily aggregates (Giasson *et al.*, 2001). These results are not

necessarily in disagreement with those of Bodles *et al.*, as the two fragments have a considerable overlap. Interestingly, site-directed spin labeling coupled to electron paramagnetic resonance spectroscopy has shown that the β -core region of α -syn fibrils is composed by a central, relatively large portion of α -syn (Der-Sarkissian *et al.*, 2003). This region, which includes the whole NAC region and spans residues 34–101 (Der-Sarkissian *et al.*, 2003), extends well beyond the 68–78 (or 71–82) region identified by sequence dissection studies. It is likely that the 68–78 (or 71–82) region initiates the process of aggregation that subsequently extends to other portions of the NAC sequence and even further.

3.1.3. PHF43 (Fragment from τ)

The τ protein exists as a number of isoforms with variable lengths. These are known to form fibrillar aggregates, termed paired helical filaments (PHFs), under pathological conditions such as Alzheimer's disease and frontotemporal dementia with Parkinsonism (Spillantini and Goedert, 2001; Selkoe, 2003). Regardless of the isoform, the core of the PHFs is formed by a central portion of τ , named the “repeat region” (Jakes *et al.*, 1991). Further dissection of the repeat region within the fetal isoform of τ has led to the identification of a 43-residue peptide (PHF43), as a highly amyloidogenic portion of the protein (von Bergen *et al.*, 2000). The sequence motif that supports the aggregation of PHF43, identified with a spot membrane-binding assay and consisting of the ³⁰⁶VQIVYK³¹¹ sequence (von Bergen *et al.*, 2000), corresponds to the major hydrophobic peak of PHF43 (Figure 3-2C) and to one of the major peaks of the entire τ protein.

3.1.4. AcP

AcP has served as a useful model system for investigating the fundamentals of amyloid formation (Chiti *et al.*, 2002a, 2003). Although this protein adopts a well-defined globular structure, aggregation into protofibrillar structures from a partially folded state has been studied by incubating the protein in the presence of trifluoroethanol (TFE) (Chiti *et al.*, 2002a). These solvent conditions induce the unfolding of the protein, allowing aggregation to be subsequently studied from the resulting ensemble of partially folded structures (Chiti *et al.*, 2002a). The two regions of the sequence 16–31 and 87–98 were identified as the most relevant stretches of the AcP sequence in this aggregation process, due to the observation that conservative amino acid substitutions alter the aggregation rate of the whole protein only when involving residues within these two traits (Chiti *et al.*, 2002a). Dissection of the sequence of AcP into smaller fragments have indicated that these two stretches are indeed poorly soluble, therefore confirming their important role in the aggregation of the entire protein (Chiti *et al.*, 2002a). In agreement with the three cases described above, the two regions 16–31 and 87–98 of AcP match peaks of relatively high hydrophobicity (Figure 3-2D).

3.2. Protein Aggregation Is Promoted by Hydrophobic Regions of a Sequence

The four cases analyzed here clearly show that residues promoting the aggregation of a relatively unstructured polypeptide chain are not spread all over the sequence. They cluster within relatively narrow traits of the sequence. It is therefore appropriate to envisage protein aggregation as a process promoted by well-defined regions of the sequence, rather than a set of residues distant

from each other. In this respect protein aggregation is distinct from protein folding where residues nucleating the formation of the folded structure may be relatively distant from each other along the sequence (Fersht, 1999). Another feature emerging from these studies is that aggregation-promoting regions are hydrophobic. They are crucial for triggering the aggregation process and form the core of the fibrils (Jakes *et al.*, 1991; Petkova *et al.*, 2002; Torok *et al.*, 2002; Der-Sarkissian *et al.*, 2003). It is therefore likely that such regions promote the association between protein molecules by forming the first intermolecular contacts that subsequently may propagate to other portions of the sequence.

In agreement with these observations, elongation of the C-terminus of A β_{1-40} by the two hydrophobic extra residues composing A β_{1-42} increases the propensity of the peptide to assemble into oligomers (Bitan *et al.*, 2003). In addition, amino acid substitutions within aggregation-promoting regions generally reduce the aggregation propensity of a sequence when they decrease the hydrophobicity at the site of mutation (Otzen *et al.*, 2000; Chiti *et al.*, 2002a, 2003; Wurth *et al.*, 2002). However, the effect of mutations on aggregation cannot be rationalized and predicted solely on these grounds, as other factors are equally important in this regard (see Section 5).

4. Importance of Charge in Protein Aggregation

4.1. Reduction of the Net Charge of a Protein Increases Its Propensity to Aggregate

Numerous observations indicate that the net charge is another major determinant of the aggregation behavior of a protein. RNase Sa forms ThT-positive and β -sheet-containing aggregates in the presence of denaturing concentrations of TFE (Schmittschmitt and Scholtz, 2003). The minimum solubility of the TFE-denatured protein was observed at a pH value corresponding to the isoelectric point (pI) of RNase Sa, when the protein has a charge of zero (Figure 3-3A). Two variants of RNase Sa, having respectively 3 and 5 solvent-exposed acidic residues replaced by lysine (3K and 5K RNase Sa), displayed different profiles of protein solubility versus pH, but in both cases the pH of minimum solubility equalled the pI (Figure 3-3A). These profiles are mainly attributable to the effect that pH has on global net charge rather than hydrophobicity of the polypeptide if we consider that a protein does not have a minimum number of total charged residues at the pI.

In another study, the effects of single amino acid substitutions was investigated on the propensity of TFE-denatured AcP to aggregate (Chiti *et al.*, 2002b). The mutations were designed to modify the charge state of AcP without affecting significantly the hydrophobicity or secondary structural propensities of the polypeptide chain. Although mutations decreasing the positive net charge of the protein resulted in an accelerated formation of β -sheet containing aggregates able to bind Congo red and ThT, mutations increasing the charge resulted in the opposite effect (Figure 3-3B). It has to be clarified, however, that the effect of mutations on aggregation cannot be generally rationalized and predicted solely using electrostatic arguments, given the fact that the effect of mutations on additional factors such as hydrophobicity and secondary structure propensity are also important (see Section 5). The results obtained with polyamino acids are also eloquent. Although uncharged polyglutamine and polythreonine form amyloid fibrils at neutral pH and relatively high temperatures, polylysine and polyglutamate remain soluble and unstructured under these conditions (Fandrich and Dobson, 2002). Polylysine and polyglutamate aggregate only when they are neutralized by changing the pH to values of 11 and 4, respectively, provided the temperature is maintained high (Fandrich and Dobson, 2002).

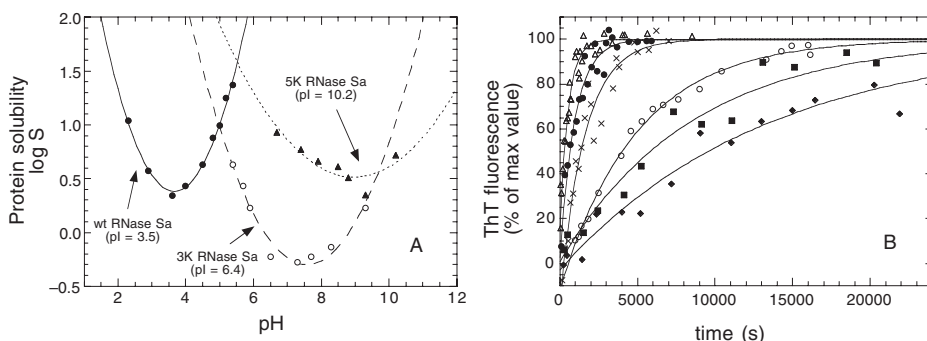


Figure 3-3. (A) Solubility as a function of pH for three variants of RNase Sa. Solubility is expressed as $\log S$, where S is solubility in mg ml^{-1} . The described variants are wild-type RNase Sa (●, continuous line), RNase Sa with three acidic residues replaced by lysines (○, dashed line), and RNase Sa with five acidic residues replaced by lysines (▲, dotted line). All samples were incubated in 30% TFE at the indicated pH before measuring solubility. Solubilities of the three variants appear to be minimal around their respective isoelectric points (pI). (Reproduced with permission from Schmittschmitt and Scholtz, 2003. Copyright 2003 Cold Spring Harbor Laboratory Press.) (B) Aggregation of six mutants of AcP followed by ThT fluorescence. The mutations were designed to change the charge of AcP while minimizing changes of hydrophobicity, α -helical, and β -sheet propensity (Chiti *et al.*, 2002b). Aggregation was initiated in each case in 25% TFE, pH 5.5, 25°C. The AcP variants shown are: wild type (●), R23Q (△), E29Q (X), E29R (○), S21R (■), and E90H (◆). Charge-increasing and charge-decreasing amino acid substitutions decelerate and accelerate aggregation of positively charged AcP, respectively. The solid lines through the data points represent the best fits to single exponential functions. (Reproduced with permission from Chiti *et al.*, 2002b. Copyright 2002 National Academy of Sciences, U.S.A.)

Unfolded or partially folded proteins appear to aggregate more readily when the numbers of positively and negatively charged amino acid residues are equal and the resulting net charge is zero. Under these conditions the repulsion between the various protein molecules is minimized, and these can approach each other more easily. The resulting particles of protein aggregates are stable due to their neutrality. By contrast, protein molecules that have a charge different from zero need to face an overall repulsion when approaching each other to aggregate. Interestingly, five residue peptides were generally found to be soluble when having a charge of ± 2 , to aggregate slowly into amyloid fibrils when displaying charge of ± 1 , and to aggregate rapidly into amorphous structures when having a net charge of zero (Lopez de la Paz *et al.*, 2002). These studies indicate that formation of ordered amyloid fibrils, as opposed to generic aggregation, requires a compromise between a too high net charge (favoring solubilization) and a too low net charge (favoring rapid and disordered aggregation).

4.2. Aggregation Is Favored by Macromolecules with an Opposite Charge

Further indications on the importance of charge in protein aggregation come from observations that aggregation of polypeptide chains is facilitated by macromolecules that exhibit a charge opposite to them. Fibrils formed from a positively charged fragment of A β , spanning residues 25–35, are able to induce aggregation of a number of soluble proteins at neutral pH (Konno, 2001). Although small

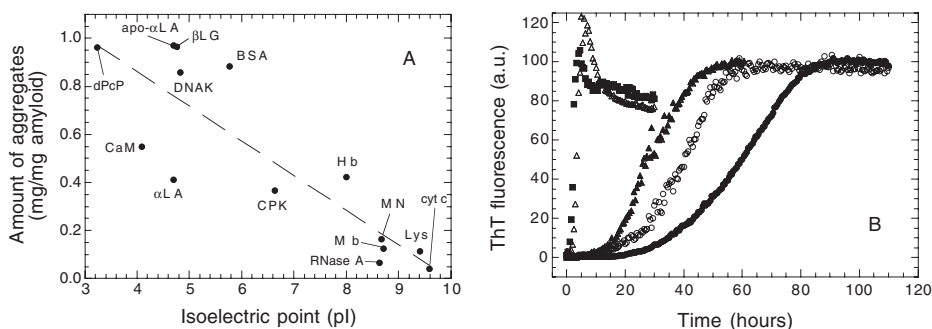


Figure 3-4. (A) Amount of precipitation of proteins induced at neutral pH by amyloid fibrils of $A\beta_{25-35}$ (expressed as ratio of total precipitate and amount of initial $A\beta_{25-35}$ fibrils) plotted against the isoelectric points (pI) of the proteins. The dashed line represents the best fit to a linear function. The proteins reported in the figure are monellin (MN, pI 6.68), horse cytochrome *c* (cyt *c*, pI 9.59), bovine ribonuclease A (RNase A, pI 8.64), human α -lactalbumin (α LA, pI 4.70), apo- α -lactalbumin (apo- α LA, pI 4.70), hen lysozyme (Lys, pI 9.41), bovine calmodulin (CaM, pI 4.09), sperm whale myoglobin (Mb, pI 8.71), bovine β -lactoglobulin A (β LG, pI 4.76), alkaline-denatured porcine pepsin (dPcP, pI 3.24), rabbit creatine phosphokinase (CPK, pI 6.63), bovine hemoglobin (Hb, pI 8.00), bovine serum albumin (BSA, pI 5.77), and DnaK (DNAK, pI 4.83). Positively charged $A\beta_{25-35}$ fibrils induce aggregation preferentially for negatively charged proteins. (Reproduced with permission from Konno, 2001. Copyright 2001 American Chemical Society.) (B) Fibrillization of α -syn (70 μ M) monitored with ThT fluorescence in the presence of 0 μ M (●), 0.45 μ M (○), 4.5 μ M (▲), 14 μ M (△), and 45 μ M (■) histone H1. Fluorescence values are normalized to the maximum value obtained using no histone H1. Addition of increasing amounts of the positively charged histone H1 accelerates aggregation of negatively charged α -syn. (Reproduced with permission from Goers *et al.*, 2003b. Copyright 2003 American Chemical Society.)

amounts of aggregates can be induced for positively charged proteins, large amounts can be formed for proteins that are negatively charged at the investigated pH (Figure 3-4A). Fibrillization of negatively charged α -syn can be induced by histones, positively charged DNA-binding proteins (Figure 3-4B), and more generally by polycations (Goers *et al.*, 2003a, 2003b). Analogously, formation of paired helical filaments from the positively charged τ is effectively facilitated in the presence of polyanions (Friedhoff *et al.*, 1998). Even more intriguingly, coincubation of τ and α -syn synergistically promote fibrillation of both proteins (Giasson *et al.*, 2003).

4.3. Charge Interactions Modulate, Rather Than Promoting Specifically, Aggregation

Although electrostatic interactions appear to play an important role in protein aggregation, all these studies show that electrostatic effects are important determinants in modulating rather than promoting specifically aggregation. Charged residues are determinants of protein aggregation because they contribute to the overall charge of a polypeptide chain, not for their involvement in specific salt bridges or repulsions between charged pairs. Amino acid substitutions increase the propensity to aggregate when they decrease the overall net charge independently of the site of mutation (Chiti *et al.*, 2002b). Similarly, progressive deviation of the pH from the isoionic point of a protein results in a gradual but continuous increase of solubility (Schmittschmitt and Scholtz, 2003). No steep deviations are observed following the titration of specific residues. In this context, electrostatic

interactions differ from hydrophobic interactions. Regions of the sequence with a high density of hydrophobic residues promote the aggregation of a protein (see Section 2.1). By contrast, electrostatic forces inhibit the aggregation process through a nonspecific repulsive effect that involves the protein molecule as a whole and is minimized when the overall net charge approaches zero.

One of the most intriguing lines of evidence in this direction comes from the structure of A β amyloid fibrils recently proposed by Tycko and coworkers (Petkova *et al.*, 2002). According to this structure, Asp23 is engaged in a stabilizing salt bridge with Lys28 (Petkova *et al.*, 2002). The conservative substitution of Asp23 with Asn, a naturally occurring replacement associated with severe cerebral amyloid angiopathy and known as the Iowa mutation, results in the suppression of the Asp23–Lys28 salt bridge, but also in the change of the charge of A β from -3 to -2 at neutral pH. The latter effect seems to be more important as D23N mutated A β forms fibrils more rapidly than the wild-type peptide *in vitro*, and is responsible for severe amyloid deposition in blood vessels from A β in patients bearing this substitution (Van Nostrand *et al.*, 2001).

It is of interest in this context to consider that proteins such as transthyretin, α -syn, and β 2-microglobulin aggregate into amyloid fibrils at pH values around 4–5, close to the isoionic points of these proteins (Lai *et al.*, 1996; McParland *et al.*, 2000; Uversky *et al.*, 2001). This behavior is generally interpreted to originate from the formation, under these conditions of pH, of partially folded states exhibiting a high amyloidogenic potential. Although formation of such conformational states is undoubtedly an important triggering event in aggregation, it is likely that a low or null charge state also contributes, at least in part, to the preferential aggregation of these systems at these pH values.

5. Importance of the Propensity to a Form Secondary Structure in Protein Aggregation

5.1. Sequences with a High Propensity to Form β -Structures Are Highly Amyloidogenic

In addition to hydrophobicity and charge, the question arises as to whether the intrinsic propensity of an amino acid sequence to form β -structures is an important factor in promoting formation of β -structured aggregates such as amyloid fibrils. Hecht and coworkers designed a combinatorial library of sequences that shared identical patterns of regularly alternating polar and nonpolar residues, in principle ideal for β -sheet structures (West *et al.*, 1999). The resulting proteins self-assembled into Congo red binding and β -sheets containing amyloid-like fibrils (West *et al.*, 1999). If such alternating periodicity was interrupted by lysine residues at positions originally occupied by nonpolar amino acids, amyloid formation was prevented and the sequences folded into β -sheets containing monomeric proteins (Wang and Hecht, 2002).

Several independent studies carried out on various systems indicate that mutations facilitate (or reduce) aggregation when they markedly increase (or decrease) the intrinsic propensity of a polypeptide to form β -structures (Moriarty and Raleigh, 1999; Li *et al.*, 2001; Chiti *et al.*, 2002a; Ciani *et al.*, 2002; Tjernberg *et al.*, 2002; Williams *et al.*, 2004). Proline-scanning mutagenesis experiments invariably show that substitution of proline for any other residue inhibits fibril formation, provided the substitution involves a position that is critical for amyloid formation (Figure 3-5A) (Moriarty and Raleigh, 1999; Williams *et al.*, 2004). This constitutes a strong indication of the importance of β -sheet propensity in aggregation after the consideration that proline residues have the lowest capability to adopt β -sheet structures among the natural amino acids (Chou and Fasman, 1978).

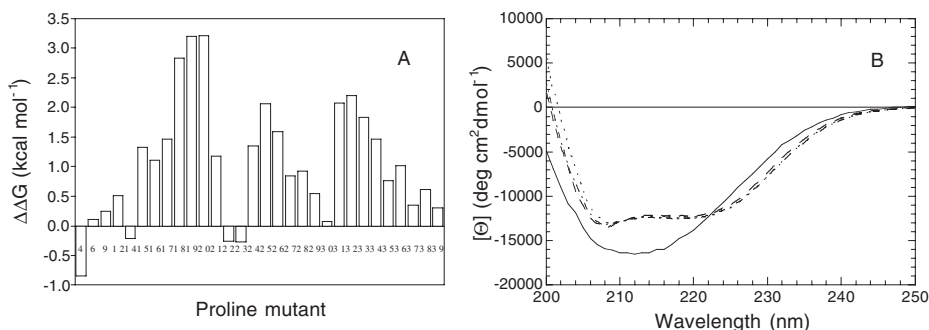


Figure 3-5. (A) Effect of proline replacement on the stability of A β_{1-40} amyloid fibrils defined as the change in ΔG for fibril formation from the monomer between the mutant and the wild-type A β . Each bar represents a mutant with the residue reported on the x axis substituted by proline. Positive and negative $\Delta\Delta G$ values indicate that the fibrils are less and more stable for the mutant than for the wild type, respectively. Substitution of a residue with the β -breaker proline residue results in a marked destabilization of the fibrils when such substitutions occur within the regions of the sequence forming the β -core structure of the fibrils. (Reprinted from Williams *et al.*, 2004. Copyright 2004, with permission from Elsevier.) (B) Far-UV CD spectra of wild-type ADA2h (continuous line), variant with a stabilized α -helix 1 (dashed line), variant with a stabilized α -helix 2 (dotted line), and variant with both α -helices stabilized (dashed and dotted line). The spectra were acquired after unfolding the protein samples at pH 3.0, 95°C and subsequently cooling down to 25°C. The spectra of the three α -helix stabilized variants are superimposable and indicate reacquisition of α -helical structures after this procedure that, by contrast, induces aggregation in wild-type ADA2h. (Reproduced with permission from Villegas *et al.*, 2000. Copyright 2000 Cold Spring Harbor Laboratory Press.)

Residues 15–21 and 31–37 of A β are recognized to form the β -core structure of A β fibrils (see Section 2.1). These two hydrophobic regions are more sensitive to proline substitution than others (Williams *et al.*, 2004). Similarly, mutations able to modulate the β -sheet propensity of AcP are more effective in changing the aggregation rate of this protein when they occur within hydrophobic and aggregation-promoting traits of the sequence (Chiti *et al.*, 2002a). This suggests that β -sheet propensity is a more effective determinant of aggregation within aggregation-promoting stretches. Residues with the highest β -propensity are β -branched amino acids, such as valine, isoleucine, tyrosine, and phenylalanine. Such residues are also hydrophobic. Consequently, aggregation-promoting regions such as those indicated for four sample sequences in Figure 3-2, match peaks of both hydrophobicity and β -propensity in the corresponding profiles (data not shown). We cannot therefore exclude that regions found experimentally to promote aggregation do so because of their high propensity to form β -structures in addition to their relatively high hydrophobicity. Distinction between these two determinants will need protein engineering methods that finely tune one factor independently of the other.

5.2. Sequences with a High Propensity to Form α -Helical Structures Exhibit Poor Amyloidogenicity

If, on the one hand, a high β -sheet propensity appears to favor aggregation, several studies indicate, on the other, that propensity to aggregate and form amyloid fibrils diminishes following

single or multiple mutations aimed at increasing selectively α -helical propensity (Soto *et al.*, 1995; Villegas *et al.*, 2000; Kallberg *et al.*, 2001; Taddei *et al.*, 2001). The activation domain of procarboxypeptidase A2 (ADA2h), for example, form β -sheet containing amyloid fibrils upon denaturation at low pH and high temperature and subsequent cooling (Villegas *et al.*, 2000). Variants containing a series of mutations designed to increase the local stability of each of the two helical regions of the protein have been found to refold to their native state upon this treatment (Figure 3-5B) (Villegas *et al.*, 2000). Uncharged polyglutamic acid and polylysine, neutralized at pH 4 and 11, respectively, do not aggregate until the α -helical structure formed under these conditions is destabilized by raising the temperature (Fandrich and Dobson, 2002).

The presence in a protein of one or more α -helices with very low propensity to adopt α -helical structures is suggested to be an indicator of the amyloidogenicity of the entire protein (Kallberg *et al.* 2001). Because TFE, a commonly used α -helical inducer, was shown to speed up fibril formation of A β , it was suggested that accumulation of a conformational state with considerable α -helical structures is a necessary transition before fibril formation (Fezoui and Teplov, 2002). This contrasts with the numerous observations that amino acid substitutions that increase α -helical propensity tend rather to inhibit aggregation. One possible explanation to this discrepancy is that, in addition to inducing α -helical structures, TFE has many other effects on polypeptide chains, including stabilization of β -sheet hydrogen bonds and exposure of hydrophobic patches to the solvent, both favoring aggregation (Buck, 1998). Stabilization of α -helical structures guided by protein engineering methods is devoid of such complicating side effects.

6. Mutations Modulate Aggregation as a Result of Their Effects on Simple Physicochemical Determinants

The proposed generic ability of polypeptide chains to aggregate into amyloid fibrils sharing common structural characteristics and the increasing evidence that simple physicochemical factors are key determinants of this process have encouraged us to investigate whether the effects of amino acid substitutions on the propensity of proteins to aggregate can be described by relatively simple principles of general validity. As described in Section 2.1, a detailed mutational analysis of AcP has allowed the regions of the sequence that promote aggregation of the denatured state of this protein to be identified (Chiti *et al.*, 2002a). After these regions were mapped out along the sequence of AcP, additional variants have been produced, all having single amino acid substitutions within them. Three statistically significant correlations were found between the change of aggregation rate resulting from mutation [$\ln(v_{\text{mut}}/v_{\text{wt}})$] and the change of (1) hydrophobicity, (2) propensity to convert from α -helical to β -structures, and (3) charge following substitution (Chiti *et al.*, 2003). Although the first two correlations were found using mutations that do not involve change of charge upon mutation, the third was investigated using mutations that aimed at minimizing changes of hydrophobicity and propensity to form α - or β -structures. This allowed double counting of factors in the three correlations to be considerably reduced. A phenomenological formula that expresses $\ln(v_{\text{mut}}/v_{\text{wt}})$ as a function of all these factors was edited. This was shown to have a satisfactory predictive power, provided aggregation is studied from an unstructured system and the mutation involves a residue within an aggregation-promoting region of the sequence. The formula was found to predict with satisfactory accuracy the effect of mutations on the aggregation rate of denatured AcP as well as other unstructured systems, including A β , α -syn, τ , and the islet amyloid polypeptide (IAPP) (Figure 3-6).

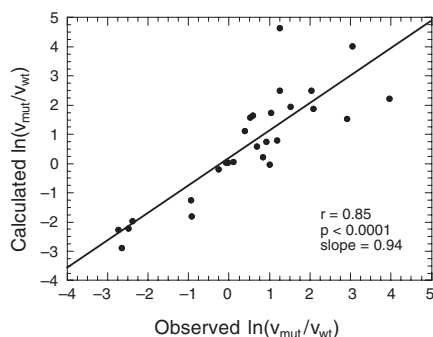


Figure 3-6. Calculated versus observed change of the aggregation rate upon mutation. Data points represent mutations of short peptides or natively unfolded proteins, including IAPP, A β , τ , and α -syn. The 27 mutations shown in the plot, along with their *calculated* and *experimental* $\ln(v_{\text{mut}}/v_{\text{wt}})$ values, are tabulated (Chiti *et al.*, 2003). The references from which the experimental $\ln(v_{\text{mut}}/v_{\text{wt}})$ values were obtained are also reported (Chiti *et al.*, 2003). The *calculated* $\ln(v_{\text{mut}}/v_{\text{wt}})$ values were obtained using an equation that expresses $\ln(v_{\text{mut}}/v_{\text{wt}})$ resulting from mutation as a function of the change of hydrophobicity, propensity to convert from α -helical to β -structures and charge following mutation (Chiti *et al.*, 2003). The significance of the correlation indicates that the effects of mutations on the aggregation of unstructured systems can be rationalized to a good approximation using simple physicochemical determinants. (Reproduced with permission from Chiti *et al.*, 2003. Copyright 2003 Nature Publishing Group.)

7. Amino Acid Sequences Have Evolved to Take into Account the Influence of Hydrophobicity, Charge, and β -Sheet Propensity in Protein Aggregation

Has an evolutionary pressure existed to select against protein sequences with a high propensity to aggregate? If simple determinants such as hydrophobicity, charge, and propensity to form secondary structures are so important, the question arises as to whether amino acid sequences have evolved to escape aggregation by tuning these factors. Folding into globular structures is an effective strategy for proteins to escape aggregation in addition to generating specific biological functions (Dobson, 2003). In this conformational state most of the hydrophobic residues of a protein are buried. Many amide and carbonyl groups are also engaged in the formation of α -helices and/or intramolecular β -sheet structures, and therefore shielded from the possibility of forming protein–protein interactions. Charges are exposed to the solvent, therefore promoting their repulsive action when the protein molecules have a net charge different from zero.

Nevertheless, even folded proteins retain a significant susceptibility to aggregate. First, they can transiently adopt unfolded or partially unfolded conformations, particularly during biosynthesis, under stress conditions or as a consequence of proteolysis or mutation. Second, even in a fully folded state proteins can aggregate, for example, through the intermolecular interactions of β -strands located at the edges of individual β -sheets (Richardson and Richardson, 2002). A survey of a large data set of all- β protein structures revealed that edge β -strands are covered with α -helices or loops or are particularly short, have strategically placed charges, β -bulges, or prolines (Richardson and Richardson, 2002). These structural adaptations contribute to dramatically decrease the β -sheet propensity of these potentially sticky β -strands, and hence, inhibit interactions between distinct

folded molecules via a possible interaction of their β -sheets (Richardson and Richardson, 2002). Along these lines, patterns of alternating hydrophilic and hydrophobic residues, shown to favor self-assembly of proteins into amyloid fibrils because of their propensity to form β -structures (see Section 4.1), are less frequent in natural proteins than expected on a purely random basis (Broome and Hecht, 2000). Furthermore, it has been suggested that the high conservation of proline residues in a fibronectin type III superfamily can be rationalized on the grounds that proline residues prevent aggregation by interfering with the formation of β -sheet structures (Steward *et al.*, 2002).

In addition to select against sequences with a dangerously high β -sheet propensity, proteins have also evolved to minimize clusters of hydrophobic residues. Groups of three or more consecutive hydrophobic residues are less frequent in natural protein sequences than expected if no selection was present (Schwartz *et al.*, 2001). Comparison of large data sets of natively unfolded and natively folded proteins has shown that the former have an hydrophobic content lower and a net charge higher than the latter (Uversky, 2002). This contributes to maintain the aggregation propensity of natively unfolded proteins particularly low as these systems are in principle highly susceptible to form aggregates due to exposure of reactive groups.

8. Other Factors Involved in Protein Aggregation

Many other factors, in addition to the simple physicochemical factors described above, can determine and modulate the amyloidogenicity of a polypeptide chain. The conformation adopted by a protein is undoubtedly a crucial factor for its ability to aggregate. Folded proteins increase dramatically their tendency to aggregate when exposed to mild denaturing conditions that cause them to unfold partially and adopt a partially folded state (Lai *et al.*, 1996; McParland *et al.*, 2000; Chiti *et al.*, 2002a; Stefani and Dobson, 2003; Uversky and Fink, 2004). In addition, mutations causing the folded state to be destabilized increase the tendency of a protein to aggregate (Hurle *et al.*, 1994; Booth *et al.*, 1997; Chiti *et al.*, 2000; Ramirez-Alvarado *et al.*, 2000; Hammarstrom *et al.*, 2002; DiDonato *et al.*, 2003; Smith *et al.*, 2003). It has been shown that natively unfolded proteins also need to acquire a partially folded state to aggregate, although such a conformational state is far less structured than the folded state of a typically globular protein (Lee *et al.*, 1995; Uversky, *et al.* 2001; Uversky and Fink, 2004). Anions induce partial folding of α -syn at neutral pH, forming a critical amyloidogenic intermediate that leads to significant acceleration of the rate of fibrillation (Munishkina *et al.*, 2004). The relationship between protein three-dimensional structures and aggregation is presented and discussed in more detail in Chapter 1.

Cu^{2+} and Zn^{2+} ions are also emerging as key determinants in protein aggregation (Bush, 2003). The delicate role that Cu^{2+} and Zn^{2+} ions play in amyloid formation cannot be framed within relatively simple electrostatic arguments. It has also been proposed that interactions between aromatic rings of amino acid residues could be essential in amyloid aggregation (Azriel and Gazit, 2001). This view has been prompted by the finding that replacement of phenylalanine with alanine in the hexapeptide NFGAIL, corresponding to residues 22–27 of IAPP, prevents its amyloid formation (Azriel and Gazit, 2001). However, the importance of aromatic–aromatic interactions is at present debated because fragments of IAPP having phenylalanine substituted by leucine were shown to retain a similar ability to form amyloid fibrils (Tracz *et al.*, 2004).

Following limited-proteolysis experiments, it has also been shown that the regions of the sequence that promote aggregation of a protein from its partially folded state possess a considerable flexibility and/or solvent exposure in addition to a relatively high hydrophobicity and propensity to form β -sheet structures (Monti *et al.*, 2004). It is therefore becoming clear that the aggregation of a

partially unstructured polypeptide chain is also modulated by the accessibility of key stretches to the solvent and interactions with other protein molecules. A comprehensive list of all factors that were shown to determine the aggregation properties of polypeptide chains cannot be presented and described exhaustively in a single paragraph. The factors described in this paragraph, to which we can add the involvement of redox status of cysteine and methionine residues (Uversky *et al.*, 2002) and the formation of off-pathway oligomers (Yamin *et al.*, 2003) are just a few examples that many factors determine the aggregation behavior of polypeptide chains, in addition to the simple physicochemical determinants that represent the focus of this chapter.

9. Is Protein Aggregation Driven by Specific Sequences?

Some investigators have proposed that specific sequences act as signature motifs (consensus sequences) for amyloidogenic proteins and help identify regions promoting amyloidogenesis within them (El-Agnaf and Irvine, 2000; Lopez de La Paz and Serrano, 2004). One of such proposed sequences is the GAXX motif, where X is a residue with an aliphatic side chain (El-Agnaf and Irvine, 2000). However, the GAXX is not present in the highly amyloidogenic 71–82 peptide of α -syn. It is also missing in the regions of the sequence that are critical for the aggregation of A β , τ , AcP, as well as other amyloidogenic proteins.

An alternative signature motif was proposed recently (Lopez de la Paz and Serrano, 2004). This consists of the six-residue sequence {P}₁–{PKRHW}₂–[VLSCWFNQ]₃–[ILTYWFN]₄–[FIY]₅–[PKRH]₆, where residues in square brackets ([]) are those allowed at the position and residues indicated in curly brackets ({ }) are those forbidden at the position. Although this motif identifies regions of the sequence of amyloidogenic proteins and peptides that were found experimentally to promote aggregation (Lopez de la Paz and Serrano, 2004), this sequence is generally absent in critical regions of other amyloidogenic proteins. These discrepancies bring into question the significance of signature motifs in amyloid formation. After the proposal that amyloid formation is a generic property of polypeptide chains (see Chapter 2), this process is likely to arise from simple, nonspecific physicochemical factors, rather than promoted specifically by particular sequences.

10. Future Perspectives

The large body of experimental data described here shows that amino acid composition and sequence determine considerably the propensity of a given polypeptide chain to fibrillize. Although considerable progress has to be achieved for a full understanding of protein aggregation, the influence of side chains on protein aggregation can be described using physicochemical arguments that are probably less complex than previously thought. While we were writing this book, a number of experimentalists and theoreticians were trying to edit algorithms of generic applicability to determine, for example, the rate and/or propensity of polypeptide chains to aggregate or to identify which regions of a given sequence are involved in the aggregation process. The simple phenomenological formula described in Section 5 to determine the effect of mutations on aggregation rate is also being reanalyzed to edit more sophisticated and complete algorithms from first principles. Some of the works describing the efforts in this direction were just published (Dubay *et al.* 2004; Fernandez-Escamilla *et al.*, 2004; Tartaglia *et al.* 2004). The increasing availability of experimental data will provide chances to increase the accuracy and reliability of these algorithms. Although protein aggregation remains a challenging problem, significant advances achieved in the past 5 years suggest that an understanding of this phenomenon is hopefully within our reach.

11. Abbreviations

A β	Amyloid β peptide
AcP	Muscle acylphosphatase
α -syn	α -Synuclein
IAPP	Islet amyloid polypeptide
NAC	Nonamyloid component
NMR	Nuclear magnetic resonance
PHF	Paired helical filament
PHF43	Fragment of τ protein
TFE	Trifluoroethanol

References

- Azriel, R., and Gazit, E. (2001). Analysis of the minimal amyloid-forming fragment of the islet amyloid polypeptide. An experimental support for the key role of the phenylalanine residue in amyloid formation. *J. Biol. Chem.* 276: 34156–34161.
- Bitan, G., Kirkitadze, M.D., Lomakin, A., Vollers, S.S., Benedek, G.B., and Teplow, D.B. (2003). Amyloid β -protein (A β) assembly: A β 40 and A β 42 oligomerize through distinct pathways. *Proc. Natl. Acad. Sci. USA* 100: 330–335.
- Bodles, A.M., Guthrie, D.J., Greer, B., and Irvine, G.B. (2001). Identification of the region of non-Abeta component (NAC) of Alzheimer's disease amyloid responsible for its aggregation and toxicity. *J. Neurochem.* 78:384–395.
- Booth, D.R., Sunde, M., Bellotti, V., Robinson, C.V., Hutchinson, W.L., Fraser, P.E., Hawkins, P.N., Dobson, C.M., Radford, S.E., Blake, C.C.F., and Pepys, M.B. (1997). Instability, unfolding and aggregation of human lysozyme variants underlying amyloid fibrillogenesis. *Nature* 385:787–793.
- Bousset, L., Thomson, N.H., Radford, S.E., and Melki, R. (2002). The yeast prion Ure2p retains its native alpha-helical conformation upon assembly into protein fibrils in vitro. *EMBO J.* 17:2903–2911.
- Broome, B.M. and Hecht, M.H. (2000). Nature disfavors sequences of alternating polar and non-polar amino acids: implications for amyloidogenesis. *J. Mol. Biol.* 296:961–968.
- Buck, M. (1998). Trifluoroethanol and colleagues: cosolvents come of age. Recent studies with peptides and proteins. *Q. Rev. Biophys.* 31:297–355.
- Bush, A.I. (2003). The metallobiology of Alzheimer's disease. *Trends Neurosci.* 26:207–214.
- Chiti, F., Taddei, N., Bucciantini, M., White, P., Ramponi, G. and Dobson, C.M. (2000). Mutational analysis of the propensity for amyloid formation by a globular protein. *EMBO J.* 19, 1441–1449.
- Chiti, F., Taddei, N., Baroni, F., Capanni, C., Stefani, M., Ramponi, G. and Dobson, C.M. (2002a). Kinetic partitioning of protein folding and aggregation. *Nat. Struct. Biol.* 9:137–143.
- Chiti, F., Calamai, M., Taddei, N., Stefani, M., Ramponi, G., and Dobson, C.M. (2002b). Studies of the aggregation of mutant proteins in vitro provide insights into the genetics of amyloid diseases. *Proc. Natl. Acad. Sci. USA* 99:16419–16426.
- Chiti, F., Stefani, M., Taddei, N., Ramponi, G., and Dobson, C.M. (2003). Rationalization of the effects of mutations on peptide and protein aggregation rates. *Nature* 424:805–808.
- Chou, P.Y., and Fasman, G.D. (1978). Empirical predictions of protein conformation. *Annu. Rev. Biochem.* 47:251–276.
- Ciani, B., Hutchinson, E.G., Sessions, R.B., and Woolfson, D.N. (2002). A designed system for assessing how sequence affects alpha to beta conformational transitions in proteins. *J. Biol. Chem.* 277:10150–10155.
- Creighton, T.E. (1993). *Proteins. Structure and molecular properties*, 2nd ed. New York: W.H. Freeman & Company, p. 154.
- Der-Sarkissian, A., Jao, C.C., Chen, J., and Langen, R. (2003). Structural organization of α -synuclein fibrils studied by site-directed spin labeling. *J Biol Chem.* 278:37530–37535.
- DiDonato, M., Craig, L., Huff, M.E., Thayer, M.M., Cardoso, R.M., Kassmann, C.J., Lo, T.P., Bruns, C.K., Powers, E.T., Kelly, J.W., Getzoff, E.D., and Tainer, J.A. (2003). ALS mutants of human superoxide dismutase form fibrous aggregates via framework destabilization. *J. Mol. Biol.* 332:601–615.
- Dobson, C.M. (2003). Protein folding and misfolding. *Nature* 426:884–890.

- DuBay, K.F., Pawar, A., Chiti, F., Zurdo, J., Dobson, C.M., and Vendruscolo, M. (2004). Predicting absolute amyloid aggregation rates of polypeptide chains. *J. Mol. Biol.* 341:1317–1326.
- El-Agnaf, O.M., and Irvine, G.B. (2000). Review: formation and properties of amyloid-like fibrils derived from alpha-synuclein and related proteins. *J. Struct. Biol.* 130:300–309.
- El-Agnaf, O.M., Bodles, A.M., Guthrie, D.J., Harriott, P., and Irvine, G.B. (1998). The N-terminal region of non-A beta component of Alzheimer's disease amyloid is responsible for its tendency to assume beta-sheet and aggregate to form fibrils. *Eur. J. Biochem.* 258:157–163.
- Fandrich, M., and Dobson, C. M. (2002). The behaviour of polyamino acids reveals an inverse side chain effect in amyloid structure formation. *EMBO J.* 21:5682–5690.
- Fernandez-Escamilla, A.M., Rousseau, F., Schymkowitz, J., and Serrano, L. (2004). Prediction of sequence-dependent and mutational effects on the aggregation of peptides and proteins. *Nat. Biotechnol.* 22:1302–1306.
- Fersht, A.R. (1999). *Structure and mechanism in protein science. A guide to enzyme catalysis and protein folding.* New York: W.H. Freeman & Company, pp. 540–614.
- Fezoui, Y., and Teplow, D.B. (2002). Kinetic studies of amyloid beta-protein fibril assembly. Differential effects of alpha-helix stabilization. *J. Biol. Chem.* 277:36948–36954.
- Friedhoff, P., Schneider, A., Mandelkow, E.M., and Mandelkow, E. (1998). Rapid assembly of Alzheimer-like paired helical filaments from microtubule-associated protein tau monitored by fluorescence in solution. *Biochemistry* 37:1 0223–10230.
- Giasson, B.I., Murray, I.V., Trojanowski, J.Q., and Lee, V.M. (2001). A hydrophobic stretch of 12 amino acid residues in the middle of alpha-synuclein is essential for filament assembly. *J. Biol. Chem.* 276:2380–2386.
- Giasson, B.I., Forman, M.S., Higuchi, M., Golbe, L.I., Graves, C.L., Kotzbauer, P.T., Trojanowski, J.Q., and Lee, V.M. (2003). Initiation and synergistic fibrillization of tau and alpha-synuclein. *Science* 300:636–640.
- Goers, J., Uversky, V.N., and Fink, A.L. (2003a). Polycation-induced oligomerization and accelerated fibrillation of human alpha-synuclein in vitro. *Protein Sci.* 12:702–707.
- Goers, J., Manning-Bog, A.B., McCormack, A.L., Millett, I.S., Doniach, S., Di Monte, D.A., Uversky, V.N., and Fink, A.L. (2003b). Nuclear localization of alpha-synuclein and its interaction with histones. *Biochemistry* 42: 8465–8471.
- Hammarstrom, P., Jiang, X., Hurshman, A.R., Powers, E.T., and Kelly, J.W. (2002). Sequence-dependent denaturation energetics: a major determinant in amyloid disease diversity. *Proc. Natl. Acad. Sci. USA* 99:16427–16432.
- Hurle, M.R., Helms, L.R., Li, L., Chan, W., and Wetzel, R. (1994). A role for destabilizing amino acid replacements in light-chain amyloidosis. *Proc. Natl. Acad. Sci. USA* 91:5446–5450.
- Jakes, R., Novak, M., Davison, M., and Wischik, C.M. (1991). Identification of 3- and 4-repeat tau isoforms within the PHF in Alzheimer's disease. *EMBO J.* 10:2725–2729.
- Kallberg, Y., Gustafsson, M., Persson, B., Thyberg, J., and Johansson, J. (2001). Prediction of amyloid fibril-forming proteins. *J. Biol. Chem.* 276:12945–12950.
- Kayed, R., Bernhagen, J., Greenfield, N., Sweimeh, K., Brunner, H., Voelter, W., and Kapurniotu, A. (1999). Conformational transitions of islet amyloid polypeptide (IAPP) in amyloid formation in vitro. *J. Mol. Biol.* 287:781–796.
- Kelly, J.W. (1998). The alternative conformations of amyloidogenic proteins and their multi-step assembly pathways. *Curr. Opin. Struct. Biol.* 8:101–106.
- Konno, T. (2001). Amyloid-induced aggregation and precipitation of soluble proteins: an electrostatic contribution of the Alzheimer's beta (25–35) amyloid fibril. *Biochemistry* 40:2148–2154.
- Lai, Z., Colon, W., and Kelly, J.W. (1996). The acid-mediated denaturation pathway of transthyretin yields a conformational intermediate that can self-assemble into amyloid. *Biochemistry* 35:6470–6482.
- Laurine, E., Gregoire, C., Fandrich, M., Engemann, S., Marchal, S., Thion, L., Mohr, M., Monsarrat, B., Michel, B., Dobson, C.M., Wanker, E., Erard, M., and Verdier, J.M. (2003). Lithostathine quadruple-helical filaments form proteinase K-resistant deposits in Creutzfeldt-Jakob disease. *J. Biol. Chem.* 278:51770–51778.
- Lee, J.P., Stimson, E.R., Ghilardi, J.R., Mantyh, P.W., Lu, Y.A., Felix, A.M., Llanos, W., Behbin, A., Cummings, M., Van Crielinge, M., et al. (1995). ¹H NMR of A beta amyloid peptide congeners in water solution. Conformational changes correlate with plaque competence. *Biochemistry* 34:5191–5200.
- Li, J., Uversky, V.N., and Fink, A.L. (2001). Effect of familial Parkinson's disease point mutations A30P and A53T on the structural properties, aggregation, and fibrillation of human alpha-synuclein. *Biochemistry* 40:11604–11613.
- Lopez de la Paz, M.L., and Serrano, L. (2004). Sequence determinants of amyloid fibril formation. *Proc. Natl. Acad. Sci. USA* 101:87–92.
- Lopez De La Paz, M., Goldie, K., Zurdo, J., Lacroix, E., Dobson, C.M., Hoenger, A., and Serrano, L. (2002). De novo designed peptide-based amyloid fibrils. *Proc. Natl. Acad. Sci. USA* 99:16052–16057.

- Mangione, P., Sunde, M., Giorgetti, S., Stoppini, M., Esposito, G., Gianelli, L., Obici, L., Asti, L., Andreola, A., Viglino, P., Merlini, G., and Bellotti, V. (2001). Amyloid fibrils derived from the apolipoprotein A1 Leu174Ser variant contain elements of ordered helical structure. *Protein Sci.* 10:187–199.
- McParland, V.J., Kad, N.M., Kalverda, A.P., Brown, A., Kirwin-Jones, P., Hunter, M.G., Sunde, M., and Radford, S.E. (2000). Partially unfolded states of β 2-microglobulin and amyloid formation in vitro. *Biochemistry* 39: 8735–8746.
- Monti, M., Garolla di Bard, B.L., Calloni, G., Chiti, F., Amoresano, A., Ramponi, G., and Pucci, P. (2004). The regions of the sequence most exposed to the solvent within the amyloidogenic state of a protein initiate the aggregation process. *J. Mol. Biol.* 336:253–262.
- Moriarty, D.F., and Raleigh, D.P. (1999). Effects of sequential proline substitutions on amyloid formation by human amylin20–29. *Biochemistry* 38:1811–1818.
- Munishkina, L.A., Henriques, J., Uversky, V.N., and Fink, A.L. (2004). Role of protein–water interactions and electrostatics in alpha-synuclein fibril formation. *Biochemistry* 43:3289–3300.
- Otzen, D.E., Kristensen, O., and Oliveberg, M. (2000). Designed protein tetramer zipped together with a hydrophobic Alzheimer homology: a structural clue to amyloid assembly. *Proc. Natl. Acad. Sci. USA* 97:9907–9912.
- Petkova, A.T., Ishii, Y., Balbach, J.J., Antzutkin, O.N., Leapman, R.D., Delaglio, F. and Tycko, R. (2002). A structural model for Alzheimer's beta-amyloid fibrils based on experimental constraints from solid state NMR. *Proc. Natl. Acad. Sci. USA* 99:16742–16747.
- Plakoutsi, G., Stefani, M., Taddei, N., and Chiti, F. (2004). Aggregation of the acylphosphatase from *S. solfataricus*. The folded and partially unfolded states can be both precursors for amyloid formation. *J. Biol. Chem.* 279: 14111–14119.
- Ramirez-Alvarado, M., Merkel, J.S., and Regan, L. (2000). A systematic exploration of the influence of the protein stability on amyloid fibril formation in vitro. *Proc. Natl. Acad. Sci. USA* 97:8979–8984.
- Richardson, J.S., and Richardson, D.C. (2002). Natural β -sheet proteins use negative design to avoid edge-to-edge aggregation. *Proc. Natl. Acad. Sci. USA* 99:2754–2759.
- Rochet, J.C., and Lansbury, P.T., Jr. (2000). Amyloid fibrillogenesis: themes and variations. *Curr. Opin. Struct. Biol.* 10:60–68.
- Roseman, M.A. (1988). Hydrophilicity of polar amino acid side-chains is markedly reduced by flanking peptide bonds. *J. Mol. Biol.* 200:513–522.
- Schmittschmitt, J.P., and Scholtz, J.M. (2003). The role of protein stability, solubility, and net charge in amyloid fibril formation. *Protein Sci.* 12:2374–2378.
- Schwartz, R., Istrail, S. and King, J. (2001). Frequencies of amino acid strings in globular protein sequences indicate suppression of blocks of consecutive hydrophobic residues. *Protein Sci.* 10:1023–1031.
- Selkoe, D.J. (2003). Folding proteins in fatal ways. *Nature* 426:900–904.
- Smith, D.P., Jones, S., Serpell, L.C., Sunde, M., and Radford, S.E. (2003). A systematic investigation into the effect of protein destabilisation on beta 2-microglobulin amyloid formation. *J. Mol. Biol.* 330:943–954.
- Soto, C., Castano, E.M., Frangione, B., and Inestrosa, N.C. (1995). The alpha-helical to beta-strand transition in the amino-terminal fragment of the amyloid beta-peptide modulates amyloid formation. *J. Biol. Chem.* 270: 3063–3067.
- Spillantini, M.G., and Goedert, M. (2001). Tau gene mutations and tau pathology in frontotemporal dementia and parkinsonism linked to chromosome 17. *Adv. Exp. Med. Biol.* 487:21–37.
- Staniforth, R.A., Giannini, S., Higgins, L.D., Conroy, M.J., Hounslow, A.M., Jerala, R., Craven, C.J., and Waltho, J.P. (2001). Three-dimensional domain swapping in the folded and molten-globule states of cystatins, an amyloid-forming structural superfamily. *EMBO J.* 20:4774–4781.
- Stefani, M., and Dobson, C.M. (2003). Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. *J. Mol. Med.* 81:678–699.
- Steward, A., Adhya, S. and Clarke, J. (2002). Sequence conservation in Ig-like domains: the role of highly conserved proline residues in the fibronectin type III superfamily. *J. Mol. Biol.* 318:935–940.
- Taddei, N., Capanni, C., Chiti, F., Stefani, M., Dobson, C.M., and Ramponi, G. (2001). Folding and aggregation are selectively influenced by the conformational preferences of the alpha-helices of muscle acylphosphatase. *J. Biol. Chem.* 276:37149–37154.
- Tartaglia, G.G., Cavalli, A., Pellarin, R., and Caffisch, A. (2004). The role of aromaticity, exposed surface, and dipole moment in determining protein aggregation rates. *Protein Sci.* 13:1939–1941.
- Thuncke, F., and Fischer, G. (1998). Separation of *cis/trans* conformers of human and salmon calcitonin by low temperature capillary electrophoresis. *Electrophoresis* 19:288–294.

- Tjernberg, L.O., Callaway, D.J., Tjernberg, A., Hahne, S., Lilliehook, C., Terenius, L., Thyberg, J., and Nordstedt, C. (1999). A molecular model of Alzheimer amyloid beta-peptide fibril formation. *J. Biol. Chem.* 274:12619–12625.
- Tjernberg, L., Hosia, W., Bark, N., Thyberg, J., and Johansson, J. (2002). Charge attraction and beta propensity are necessary for amyloid fibril formation from tetrapeptides. *J. Biol. Chem.* 277:43243–43246.
- Torok, M., Milton, S., Kaye, R., Wu, P., McIntire, T., Glabe, C.G., and Langen, R. (2002). Structural and dynamic features of Alzheimer's Aβ peptide in amyloid fibrils studied by site-directed spin labeling. *J. Biol. Chem.* 277:40810–40815.
- Tracz, S.M., Abedini, A., Driscoll, M., and Raleigh, D.P. (2004). Role of aromatic interactions in amyloid formation by peptides derived from human amylin. *Biochemistry*, in press.
- Uversky, V.N. (2002). Natively unfolded proteins: a point where biology waits for physics. *Protein Sci.* 11:739–756.
- Uversky, V.N., and Fink, A.L. (2002). Amino acid determinants of alpha-synuclein aggregation: putting together pieces of the puzzle. *FEBS Lett.* 522:9–13.
- Uversky, V.N., and Fink, A.L. (2004). Conformational constraints for amyloid fibrillation: the importance of being unfolded. *Bioch. Biophys. Acta* 1698:131–153.
- Uversky, V.N., Li, J., and Fink, A.L. (2001). Evidence for a partially folded intermediate in alpha-synuclein fibril formation. *J. Biol. Chem.* 276:10737–10744.
- Uversky, V.N., Yamin, G., Souillac, P.O., Goers, J., Glaser, C.B., and Fink, A.L. (2002). Methionine oxidation inhibits fibrillation of human alpha-synuclein in vitro. *FEBS Lett.* 517:239–244.
- Van Nostrand, W.E., Melchor, J.P., Cho, H.S., Greenberg, S.M., and Rebeck, G.W. (2001). Pathogenic effects of D23N Iowa mutant amyloid β-protein. *J. Biol. Chem.* 276:32860–32866.
- Villegas, V., Zurdo, J., Filimonov, V.V., Aviles, F.X., Dobson, C.M., and Serrano, L. (2000). Protein engineering as a strategy to avoid formation of amyloid fibrils. *Protein Sci.* 9:1700–1708.
- von Bergen, M., Friedhoff, P., Biernat, J., Heberle, J., Mandelkow, E.M. and Mandelkow, E. (2000). Assembly of tau protein into Alzheimer paired helical filaments depends on a local sequence motif (³⁰⁶VQIVYK³¹¹) forming β structure. *Proc. Natl. Acad. Sci. USA* 97:5129–5134.
- Wang, W., and Hecht, M.H. (2002). Rationally designed mutations convert de novo amyloid-like fibrils into monomeric beta-sheet proteins. *Proc. Natl. Acad. Sci. USA* 99:2760–2765.
- West, M.W., Wang, W., Patterson, J., Mancias, J.D., Beasley, J.R., and Hecht, M.H. (1999). De novo amyloid proteins from designed combinatorial libraries. *Proc. Natl. Acad. Sci. USA* 96:11211–11216.
- Williams, A.D., Portelius, E., Kheterpal, I., Guo, J.T., Cook, K.D., Xu, Y., and Wetzel, R. (2004). Mapping Aβ amyloid fibril secondary structure using scanning proline mutagenesis. *J. Mol. Biol.* 16:833–842.
- Wurth, C., Guimard, N.K., and Hecht, M.H. (2002). Mutations that reduce aggregation of the Alzheimer's Aβ42 peptide: an unbiased search for the sequence determinants of Aβ amyloidogenesis. *J. Mol. Biol.* 319: 1279–1290.
- Yamin, G., Uversky, V.N., and Fink, A.L. (2003). Nitration inhibits fibrillation of human alpha-synuclein in vitro by formation of soluble oligomers. *FEBS Lett.* 542:147–152.

Cytotoxic Intermediates in the Fibrillation Pathway: A β Oligomers in Alzheimer's Disease as a Case Study

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1. Abstract

Multiple diseases, as diverse as diabetes and mad cow disease, exhibit accumulations of abnormal protein fibrils. Generically referred to as “amyloid,” these self-assembling fibrils typically have been considered the pathogenic molecules that cause cellular degeneration (toxins, not just tombstones). A prominent example is the “amyloid cascade hypothesis” proposed for Alzheimer's disease (Hardy and Higgins, 1992). Fibrils, however, are not the only toxins generated by protein self-association, probably in some cases not even the most relevant ones. We now know of toxic subfibrillar species—soluble oligomers and protofibrils. The emerging hypothesis considered here is that these novel subfibrillar assemblies, the hidden toxins, constitute significant pathogenic molecules in diseases of fibrillogenic proteins.

Clues leading to this hypothesis have come in many instances from studies of A β (Klein *et al.*, 2001), the fibrillogenic peptide responsible for amyloid plaques in Alzheimer's disease (AD). Alzheimer's disease is the most common form of dementia in the elderly, affecting 10% of individuals older than 65 (Hebert *et al.*, 2003) and more than 25 million individuals world-wide. This chapter examines the investigation of A β 's role in AD essentially as a case study. Its objectives are to (1) review the link between Alzheimer's dementia and fibrillogenic proteins and show that pathogenesis truly involves A β ; (2) show how key problems in the amyloid cascade hypothesis disappear with the discovery of subfibrillar A β assemblies; (3) discuss cellular mechanisms of the new toxins that explain why AD is a disease of memory loss; (4) consider data that clinically substantiate a new, oligomer-initiated amyloid cascade hypothesis; (5) assess whether the impact of subfibrillar toxins can provide a broad mechanism applicable to multiple fibrillogenic proteins; (6) evaluate emerging implications for therapeutics and diagnostics.

At present, no cause for AD has been established; there are no effective therapeutics, and no clinical diagnostics exist. This situation, however, is rapidly changing. New insights into disease mechanisms and remarkable new therapeutic antibody strategies give us cause for optimism.

2. AD Is a Dementia Involving the Fibrillogenic A β Peptide

At its outset, AD is almost purely a disease of crippling memory loss (Selkoe, 2002). It first blocks the ability to form new memories and later blocks retrieval of stored ones. The experience of AD dementia is hard to imagine, but a sense of its devastation comes from the story of Auguste D,

a woman in her late forties whom Alois Alzheimer treated a century ago (Alzheimer, 1907). Working with this first AD patient, Alzheimer once asked Auguste D to write her name. Unable to do so, she said, "I have lost myself . . ." her nonscientific words revealing the real meaning of AD. With time, dementia grows inexorably severe, leaving patients in a vegetative state (Coyle, 1987). The disease also accelerates the end of life, with AD and its complications representing the fourth leading cause of death. Auguste D, whose early onset probably reflected the more virulent familial AD, died in her mid-fifties. Life span of patients with sporadic AD is variable and increasing. President Ronald Reagan lived 20 years following his initial diagnosis.

The neuropathology of AD is complex and includes brain inflammation, shrinkage of the hippocampus and cerebral cortex, and degeneration of specific neuronal populations (Terry, 1999). The primary pathology linked to AD, however, comprises two types of insoluble proteinaceous deposits, first codified by Alzheimer's findings with brain tissue from Auguste D. Work from the last 20 years has determined that these deposits comprise (1) extracellular polymers of the amyloid beta peptide ($A\beta$), which make up AD's "plaques," and (2) intraneuronal polymers of hyperphosphorylated tau, which make up AD's "tangles." Presence of "plaques and tangles" in higher cognitive brain regions of a demented patient provides the definitive diagnosis of AD (Braak and Braak, 1991).

Accumulation of fibrillar $A\beta$ in plaques underlies the dominant theory for AD—the "amyloid cascade hypothesis." The hypothesis, formalized in its original form by Hardy and Higgins in 1992 (Hardy and Higgins, 1992), has been extraordinarily fertile, with reference to $A\beta$ found in over 10,000 papers. Two key questions can be asked: (1) Why has the hypothesis been so compelling? And (2) Why has it not been accepted?

Strong support for the amyloid cascade hypothesis comes from pathology, human genetics, biochemistry, and cell biology. Twenty years ago, the key constituent of amyloid fibrils was identified by Glenner and Wong (1984) as a ~4kDa peptide now called $A\beta$. Monomeric $A\beta$ is a physiological peptide that derives proteolytically from amyloid precursor protein (APP), a single-span transmembrane protein. $A\beta$ contains part of the APP transmembrane domain in tandem with a portion of the juxtamembrane domain. The extent of the membrane domain found in $A\beta$ is variable, producing peptides of 39–43 amino acids. Most common is the 40-amino acid species. Physiological proteolysis of APP is complex and highly regulated (da Cruz e Silva EF and da Cruz e Silva OA, 2003), and mutations in APP that are highly conserved increase $A\beta$ production. Most significantly, these APP mutations will cause AD (Chartier-Harlin *et al.*, 1991; Goate *et al.*, 1991).

Although APP mutations are rare, their discovery provided a breakthrough in understanding disease mechanism. APP-linked familial AD is indistinguishable from sporadic AD, the most common form of the disease; it also is identical to that caused by mutations in presenilins, which underlie a somewhat more frequent familial AD (Price *et al.*, 1998). Comparison of dementia, pathology, and metabolic consequences among the various etiologies indicates the common denominator is elevated production of the 42-amino acid form of $A\beta$ (Borchelt *et al.*, 1996). The particular peptide form is relevant because $A\beta$ 42 readily makes fibrils, especially in comparison to the less hydrophobic and much more abundant $A\beta$ 40. When anomalously induced in brain by mutations or other disease-linked factors, elevated $A\beta$ 42 precipitates as insoluble deposits of amyloid fibrils. *Ex vivo* experiments have pointed to the pathogenic significance of these insoluble fibrils. First, fibrils were found to form *in vitro* from synthetic $A\beta$ (Pike *et al.*, 1991; Hilbich *et al.*, 1991; Burdick *et al.*, 1992), essentially mimicking disease pathology and providing a tool for toxicology. Second, fibrillar preparations applied to cultured neurons exert an impact clearly relevant to AD pathogenesis: they induce AD-like tau phosphorylation (Lambert *et al.*, 1994), implicating amyloid fibrils in the generation of AD's diagnostic tangles, and, most significantly, they kill CNS neurons (Pike *et al.*, 1991, 1993; Lorenzo and Yankner, 1994).

Data from multiple disciplines thus show amyloid fibrils to be self-assembled neurotoxins generated in the AD brain by elevated levels of monomeric A β 42, a peptide firmly linked by pathology and genetics to disease mechanism. Such lines of evidence provided a firm footing for the fundamental principles of the original amyloid cascade hypothesis: (1) the primary molecular pathogen in AD was presumed to be the insoluble amyloid fibril, and (2) AD dementia was presumed to derive from fibril-induced nerve cell death.

3. Why the Fibril-Based Cascade Hypothesis Unraveled: A Singular Illustration with a Transgenic Mouse AD Model

Despite its great theoretical and experimental appeal, the amyloid cascade hypothesis in its original formulation has proven to be a flawed concept, never attaining a consensus for its acceptance. Epitomizing the fundamental flaws in the original amyloid cascade hypothesis are results from an extraordinary experimental vaccine study by Dodart *et al.* (2002).

The Dodart protocol administered monoclonal antibodies against A β to transgenic (tg) mice carrying the hAPP gene. These mice provide a powerful model of early AD, manifesting age-dependent appearance of amyloid plaques along with age-dependent memory loss. Dodart's study produced two significant findings, each inconsistent with the original amyloid cascade hypothesis. First, vaccinated animals exhibited reversal of their memory loss, with recovery evident in as little as 24 hours. Reversibility cannot be reconciled with a mechanism for memory loss based on nerve cell death. Second, therapeutic benefits of the anti-A β antibodies accrued despite no loss of amyloid plaques. Thus, the amyloid fibrils were not the pathogenic agents. Kotilinek *et al.* (2002) independently confirmed that memory loss is reversible and plaque-independent in studies with different mice and different A β antibodies. Earlier, prophylactic vaccine studies also had indicated memory loss was fibril-independent (Janus *et al.*, 2000; Morgan *et al.*, 2000).

Results with the experimental A β vaccines cannot be explained by the original amyloid cascade hypothesis. They are consistent, however, with earlier concepts regarding AD pathology and plaque independence. Scheibel, for example, suggested almost 30 years ago that neuron death may be less significant for dementia than structural changes in neuronal morphology relevant to synaptic function (Scheibel and Tomiyasu, 1978). Neuropathologists, moreover, have long raised concerns over poor correlations between amyloid plaque burden and AD dementia (Katzman *et al.*, 1988; Terry, 1994). With respect to plaque burden in AD models, a lack of correlation between amyloid and brain dysfunction caused many early tg hAPP strains to be rejected as inappropriate models of AD (Klein *et al.*, 2001). In fact, they may have been excellent models of early AD dementia, but one caused by subfibrillar A β toxins, not amyloid fibrils.

4. If Not Fibrils, What? Discovery of A β 's Hidden Toxins

Molecular-level experiments identified another crucial failing of the amyloid cascade, a finding that ultimately led to discovery of the cascade's missing link. First of all, early benchmark discoveries by Pike *et al.* (1993) and Yankner *et al.* (Lorenzo and Yankner, 1994) had established that synthetic A β preparations could kill central nervous system neurons, but only after A β had self-associated. Initially, it appeared that the structural state required for toxicity was fibrillar, a reasonable inference given the abundance of fibrils in toxic preparations along with the diagnostic presence of fibrils in AD brain. Inhibitors of fibrillogenesis thus were expected to block neurotoxicity, a prediction that

stimulated significant effort within the pharmaceutical industry. This prediction, however, proved to be untrue.

Oda and colleagues (Oda *et al.*, 1994, 1995) reported in the mid-1990s that clusterin, an AD-upregulated protein also known as ApoJ, blocked the *in vitro* formation of large A β 42 aggregates. Clusterin therefore should have blocked neurotoxicity. However, in experiments with the PC12 pheochromocytoma cell line, clusterin-treated solutions of A β were even more toxic than untreated solutions (**Figure 4-1; see color insert**). Inhibition of A β fibrillogenesis without loss of toxicity also was observed by Butterfield and colleagues in experiments with glutamine synthetase (Aksenov *et al.*, 1996). Oda *et al.* concluded that the role of fibrillar A β in AD pathogenesis required reevaluation (Oda *et al.*, 1994).

The toxicology experiments along with the animal model studies establish an important conclusion: fibrils cannot be the only toxin produced by A β self-assembly. Other species, not as obvious as fibrils, must also exist. Additionally, these hidden toxins should carry sublethal activities, to account for the reversibility of memory loss in vaccination protocols.

Pursuit of A β assemblies formed in the presence of clusterin led to the discovery of novel subfibrillar toxins, identified as small globular A β oligomers (Lambert *et al.*, 1998). Atomic force microscopy has shown clusterin-treated preparations to be entirely free of large or small fibrillar molecules. Toxic preparations contain only globular structures with Z-heights of roughly 4–5 nm. Alternative methods of preparation, not requiring clusterin, have verified the toxins solely comprise assemblies of A β (Lambert *et al.*, 2001; Walsh *et al.*, 2002; Klein, 2002; Chromy *et al.*, 2003; Stine *et al.*, 2003).

Assessed by nondenaturing electrophoresis, the globular toxins readily enter gels, confirming the absence of fibrils, while denaturing gels show SDS-stable oligomers that range from trimer to 24-mer (Lambert *et al.*, 1998; Chromy *et al.*, 2003). In solutions prepared at 4 degrees, the predominant SDS-stable oligomers are tetramers, but in solutions boosted to 37 degrees, larger oligomers emerge, predominantly 12-mers. After their formation, oligomers appear metastable, showing no rapid conversion to fibrils (Klein, 2002; Chromy *et al.*, 2003).

5. Oligomers Have Profound Neurological Impact, Accounting for Reversibility of Memory Loss

A β oligomers are similar in size to an average soluble globular protein, and would be invisible to conventional neuropathology. Supposing a relevant neurological impact, the presence of such molecules in brain potentially could explain the poor correlation between fibril deposits and dementia. They would be, in essence, the missing links in the amyloid cascade. In fact, the relevant impact as well as the association of oligomers with AD pathology has been experimentally proven.

Neurologically, AD is characterized at its outset by a specificity for memory loss (Selkoe, 2002), and a valid molecular mechanism must account for this benchmark feature of the disease. A classic experimental paradigm for investigating memory mechanisms has been hippocampal long-term potentiation (LTP). In LTP, the electrophysiological synaptic output in response to a defined input grows larger after a brief burst of excitation (Bennett, 2000). The paradigm essentially is an electrophysiological training session, with a long-lasting potentiated synaptic output measurable for days or even longer. Long-term potentiation is not memory per se, rather a form of synaptic plasticity, but its unique properties suggest a close linkage to memory mechanisms (Malenka, 2003). Long-term potentiation has proven to be highly sensitive to A β oligomers.

Exposure of hippocampal slices to fibril-free oligomer preparations completely inhibits LTP (Lambert *et al.*, 1998; Wang *et al.*, 2002). Inhibition occurs within minutes, and stems from disrup-

tion of signal transduction rather than synaptic degeneration—electrophysiological activity in axons and synapses remains normal. Originally observed through *ex vivo* experiments, oligomer-induced loss of LTP has been confirmed in animal studies employing cerebral microinjections (Walsh *et al.*, 2002). The possibility that contaminating monomers of A β might be responsible has been ruled out through application of insulin degrading enzyme, a protease that degrades monomers but not oligomers (Walsh *et al.*, 2002).

Long-term potentiation experiments have been critical in establishing that subfibrillar assemblies of A β are neither inert nor neurologically irrelevant. Inhibition of this classic paradigm for learning and memory is profoundly relevant to the neurological dysfunction of AD. The impact, which is rapid and highly selective, also suggested an iconoclastic prediction: memory loss in early AD might prove to be reversible (Lambert *et al.*, 1998; Klein *et al.*, 2001). As discussed above, such reversibility was indeed found in tg mouse models by Dodart *et al.* (2002) and Kotilinek *et al.* (2002).

The globular A β oligomers that inhibit LTP have been referred to as ADDLs (for A β -Derived Diffusible Ligands with a dementing activity), based on their neurological impact coupled with their diffusible nature and capacity for specific targeting of cell surface proteins (next section).

6. How Oligomers Attack Neurons—A Molecular Mechanism for Why AD Is Specific for Memory Loss

The impact of oligomers on LTP is in harmony with the concept that synapse failure, prior to neuron death, underlies AD memory loss (Klein *et al.*, 2001; Selkoe, 2002). Additional strong support for this concept comes from recent cell biology experiments that reveal how oligomers associate with neurons.

It has been suggested that oligomers might insert directly into membranes (Arispe *et al.*, 1994), perhaps after partially unfolding. This suggestion is consistent with the fact that A β contains a significant hydrophobic domain. Atomic force microscopy (AFM) has shown, moreover, that A β can form structures with pore-like appearance (Chromy *et al.*, 2003; Lashuel *et al.*, 2003), while ion flux data indicate A β can generate functional pores in model membranes (Arispe *et al.*, 1993; Rhee *et al.*, 1998). Because of the ensuing ion flow, such pores in cell membranes potentially would be cytotoxic.

On the other hand, oligomers are readily water soluble and SDS stable, presumably because their hydrophobic domains are sequestered within the globular structure. This reduces the likelihood they insert readily into plasma membrane lipids. Also at odds with lipid insertion, oligomers do not attach to cells that have been trypsinized (Lambert *et al.*, 1998). Perhaps most cogently, simple insertion predicts that oligomers should attach to cells nonspecifically. This prediction has not borne out. Instead, the pattern of binding is strikingly specific and most significant.

In experiments with differentiated cell cultures, oligomers clearly bind nonrandomly (Lambert *et al.*, 2001; Gong *et al.*, 2003). Oligomers added to hippocampal cultures attach to particular neurons, not to all, while in cerebellar cultures they bind to almost no cells. This neuron-selective ligand activity presumably derives from the quaternary structure of oligomers. Supporting this idea, size-exclusion chromatography indicates ligand activity fractionates with larger oligomers (12–24 mers), not with monomers or very small oligomers (Chromy *et al.*, 2003). Most significantly, specificity at the subcellular level is striking: binding sites localize in dendritic arbors where they comprise discrete punctate clusters (Gong *et al.*, 2003). This pattern is exactly as would be predicted for synaptic localization.

Very recent experiments have validated the prediction that oligomers specifically target synapses (Lacor *et al.*, 2003). Oligomer binding sites in hippocampal neuron cultures, analyzed for colocalization with the synaptic marker PSD-95 (Allison *et al.*, 2000), occur at synapses more than 90% of the time. Not all synapses are targeted, only about 50%, further validating the predicted ligand-like binding. Specific binding implies a receptor target, but as yet no receptor for oligomers has been identified. The discovery that oligomers are ligands that attach to particular synapses provides strong support for the hypothesis that memory loss in AD is a synapse failure caused by synapto-toxic A β oligomers.

7. Immediate Consequences of Oligomer Binding: Signal Transduction Targets

What happens at synapses immediately after oligomers attach? Three broad alternatives, originally proposed for the toxicity of fibrillar A β (Yankner, 1996), apply equally well to oligomers and are under investigation. First, oligomers could generate localized ion flux via transmembrane pores (Caughey and Lansbury, 2003), as observed in model membranes (Arispe *et al.*, 1994). Second, oligomers might generate synaptically localized oxidative damage. A large number of studies have shown that oligomers as well as fibrillar forms of A β generate reactive oxygen species (Hensley *et al.*, 1995; Longo *et al.*, 2000; Mattson, 2004). Third, binding to specific toxin receptors could lead indirectly to a downstream impact on signaling pathways (Lambert *et al.*, 1998).

No matter what the trigger, it has become clear that oligomers do cause particular changes in molecular signaling pathways germane to synaptic plasticity and memory mechanisms. Early findings of Cotman and colleagues with cultures of cortical neurons showed oligomers at low doses blocked the ability of the neurotransmitter glutamate to induce CREB phosphorylation (Tong *et al.*, 2001), a signaling event implicated in gene regulation relevant to memory formation. A detailed investigation germane to the mechanism of LTP inhibition has come from Wang *et al.* (2004a), who recently undertook a pharmacological strategy to investigate the role of specific protein kinases and receptors. The ability of particular agents to abolish oligomer-induced inhibition of LTP has implicated c-Jun-terminal kinase, p38 MAP kinase, cyclin-dependent kinase 5, and metabotropic glutamate receptors in the mechanism of synapse failure. In harmony with these observations, hAPP tg mice show activation of c-Jun-terminal kinase and p38 pathways, although the impact localizes to neurons near deposits of amyloid fibrils (Savage *et al.*, 2002). A possible relationship between oligomer binding and plaque development is discussed later.

Altered synaptic signaling may be closely associated with altered synaptic structure. Oligomers bound at synapses cause abnormal induction of Arc (Lacor *et al.*, 2004), a synaptic immediate early gene normally induced by synaptically activated kinases (Ying *et al.*, 2002). Arc protein in synaptic spines appears to affect f-actin organization (Lyford *et al.*, 1995), and it influences trafficking of glutamate receptors (Rial Verde *et al.*, 2003). Arc is of special interest because long-term memory formation requires its proper, transient expression. It has been proposed that sustained Arc expression blocks memory formation (Guzowski, 2002), and, in fact, its overexpression in Arc tg mice produces poor learners (Kelly and Deadwyler, 2003). In oligomer-treated cultures, Arc is induced at synapses within minutes; its expression, however, remains elevated for hours, and Arc, in affected neurons, spreads ectopically throughout dendritic arbors (Lacor *et al.*, 2004). An appealing hypothesis is that sustained Arc expression caused by oligomers produces synapse failures that underlie AD memory loss. This hypothesis suggests two testable predictions: In an Arc-dependent manner, oligomers should (1) inhibit glutamate receptor upregulation, which is involved in synaptic plasticity, and (2)

generate abnormalities in synaptic spine structure. Current evidence links abnormal spine structure with mental deficiencies and possibly with AD (Fiala *et al.*, 2002).

8. Cascading Consequences—Can Oligomer-Induced Synapse Dysfunction Lead to Synapse Destruction and Neuron Death?

Alzheimer's disease is a progressive dementia, and its pathology extends beyond synapse failure to encompass synapse destruction and neuron death. Experiments with tg animal models and brain slice cultures indicate these degenerative phenomena could derive from oligomer toxicity.

Theoretically, oligomer-induced synapse dysfunction itself might engender synapse loss. Because oligomers inhibit LTP, but prolong LTD (long-term depression), they profoundly shift synaptic activity from higher to lower levels (Wang *et al.*, 2002). Activity levels profoundly influence synapse formation during brain development (Constantine-Paton and Cline, 1998), and it is conceivable that lowered activity due to oligomers might inhibit synaptogenesis in mature brain (adult synaptogenesis is required to replace synapses due to turnover). Alternatively, synapse turnover theoretically might be accelerated directly, triggered by oligomer-induced anomalies in spine structure, perhaps coupled to Arc overexpression. In fact, synapse loss does occur in certain hAPP tg mouse AD models (Hsia *et al.*, 1999), including an AD-like degeneration of the cholinergic phenotype (Buttini *et al.*, 2002). Synapse loss correlates with levels of the total soluble A β , not with fibrillar amyloid (Mucke *et al.*, 2000), and the mechanism involves signal transduction through a Fyn kinase pathway (Chin *et al.*, 2004). Fyn, which localizes to postsynaptic densities and regulates glutamate receptor activity (Tezuka *et al.*, 1999), has been linked to synaptic plasticity, memory formation, A β toxicity, and AD pathology (for review, see Klein, 2000).

Ultimately, oligomers destroy neuronal viability. Mature brain slice cultures exposed to oligomers for extended periods (days rather than minutes) exhibit neuron death that is regionally specific (Lambert *et al.*, 1998). Parts of the hippocampus are particularly vulnerable, while the cerebellum is spared (Kim *et al.*, 2003), as in AD. As seen with synapse loss, the oligomer-induced death also is Fyn-mediated. Whether loss of synapses might lead to death of neurons is speculative, but knockout of a postsynaptic density scaffolding protein has been reported to induce neuronal apoptosis (Gardoni *et al.*, 2002). Neuron death might be caused analogously by damage to postsynaptic density proteins by synaptically bound oligomers, perhaps by localized oxidative damage. The case for this hypothesis is weakened, however, by the absence of evidence for neuron death in tg mice models.

9. In Vivo Experimental Support for Synaptotoxic Oligomers: Data from Mouse Models of Early AD

Synthetic A β oligomers are synaptotoxins with an experimental impact germane to AD memory loss, so a critical question is whether such oligomers can form in brain. An indication that oligomers can form spontaneously *in vivo* first came from findings that oligomers accumulate in culture media of cells transfected with hAPP (Podlisny *et al.*, 1995). These oligomers later were found to be synaptotoxic (Walsh *et al.*, 2002). The fact that oligomers can be maintained in solution for days indicates, moreover, a stability sufficient for accumulation in tissue (Chromy *et al.*, 2003). Neurological consequences that are plaque-independent in hAPP tg mice support this possibility (Klein *et al.*, 2001).

Recent analysis of brain extracts has provided direct confirmation of oligomer accumulation in hAPP tg mice. Dot-blot immunoassay shows the presence of soluble oligomers that are age-

region-, and transgene-dependent (Chang *et al.*, 2003). To minimize extraction artifacts, analysis was performed on soluble fractions obtained without detergents or chemicals that might disrupt fibrillar assemblies. Interestingly, a widely used monoclonal (4G8) that does not discriminate between monomers and oligomers (Lambert *et al.*, 2001) does not detect transgene-dependent oligomers that are revealed by oligomer-selective antibodies (L. Chang, unpublished). Measurement of total pools thus provides an insensitive indicator for pathogenic forms of soluble A β .

Preliminary evidence from tg mice indicates that when oligomer levels go up, performance on a water-maze memory task goes down [memory evaluated as described by Kotilinek *et al.* (2002); oligomers measured according to Chang *et al.* (2003); data provided by Chang, Kotilinek, and Ashe]. However, overall levels of soluble oligomers are less relevant to memory loss than the extent of compromised synapses. It might be anticipated, moreover, that synaptic dysfunction must exceed some threshold level before memory loss occurs. For example, even though oligomers are present, memory would be normal with subthreshold occupancy of toxin receptors, or even with complete occupancy at a subthreshold fraction of the relevant synapses. This would be analogous to Parkinson's disease, in which motor dysfunction manifests only after 60% of dopamine-producing neurons die. The threshold concept is consistent with the idea that susceptibility to AD memory loss is influenced by an individual's "synaptic reserve" (Mesulam, 1999).

10. Clinical Validation—Oligomers in Human Brain, Elevated up to 70-Fold in AD

The crucial test of the oligomer hypothesis is whether it can be substantiated by clinical data. Are oligomers present in human brain, and do they accumulate in AD? Strong support has been obtained by Kaye *et al.* (2003), who found that diffuse, early-stage plaques in AD brain are stained by an antibody against A β oligomers, essentially as predicted by Hardy and Selkoe (2002). Thioflavin-positive dense-core plaques, which contain fibrillar A β , do not crossreact. (An intriguing aspect of the Kaye study is that their antibody appears to interact generically with oligomers made from several species of proteins, considered again later.) Another oligomer-selective antibody gives diffuse stain that surrounds neuronal cell bodies, apparently binding oligomers within dendritic arbors (Bigio, Lacor, Lambert, and Viola, unpublished).

Molecular confirmation that oligomers constitute bona fide components of AD pathology comes from recent immunoanalysis of soluble brain extracts (Gong *et al.*, 2003). Quantitatively, AD brains contain strikingly elevated levels of soluble oligomers, up to 70-fold more than in controls. These large increases occur in frontal cortex but not cerebellum (L. Chang, unpublished), consistent with the specificity of AD for memory and cognition. Structural analysis confirms that the immunoreactive species are A β oligomers, detectable by conformation-sensitive antibodies raised against synthetic oligomers and indistinguishable from synthetic molecules in 2D gels (Gong *et al.*, 2003). The most prevalent soluble oligomers are 12 mers, with an isoelectric point of 5.6, although extraction in the presence of SDS indicates the presence of additional species.

Perhaps most importantly, the soluble oligomers in AD brain extracts behave as synaptic ligands (Gong *et al.*, 2003; Lacor *et al.*, 2003). As with oligomers made *in vitro*, those obtained from brain tissue bind to mature hippocampal neurons at dendritic hot spots (**Figure 4-2; see color insert**) and colocalize with synaptic markers by confocal microscopy. The oligomeric synaptic ligands from AD brain collect in crude fractions between 10 and 100kDa, consistent with the 2D gel analysis. Water-soluble oligomers in this molecular weight range were observed earlier but were regarded as intermediates en route to fibril formation and neurologically insignificant (Kuo *et al.*, 1996). We now know that oligomers are neurologically active, and the fact that oligomers from AD brain can target

hippocampal synapses strongly supports the hypothesis that memory loss in AD is an oligomer-mediated synapse failure.

11. New “Oligomer-Driven” Amyloid Hypothesis

In the seminal 1992 version of the amyloid cascade hypothesis (Hardy and Higgins, 1992), memory loss was considered the consequence of fibril-induced nerve cell death. Like any great theory, the amyloid cascade has evolved with new discoveries. We now know that fibrils are not the only A β -derived neurotoxins—A β 42 readily forms soluble, globular oligomers that target synapses and rapidly alter the molecular machinery of memory. These oligomers, the missing links in the original hypothesis, have been incorporated into an updated cascade (Klein *et al.*, 2001; Hardy and Selkoe, 2002) that features two novel concepts: (1) AD memory loss can be caused by synapse failure, independent of nerve cell death; and (2) the pathogenic agents of synapse failure are small diffusible oligomers, not fibrils. An adaptation of the cascade presented recently by Hardy and Selkoe (2002) is shown in **Figure 4-3**; see **color insert**.

The new cascade hypothesis accounts well for key features of the disease. Most importantly, it explains why early AD is memory-specific: oligomers are toxic ligands that specifically attack synapses critical for long-term memory formation. It suggests the possibility, moreover, that mild cognitive impairment, a plaque-independent memory loss, could have the same molecular basis; also, were oligomers to stimulate abnormal tau phosphorylation, as found for fibrillar preparations (Lambert *et al.*, 1994; Busciglio *et al.*, 1995), they could play a role in frontal lobe dementia, another plaque-independent dysfunction associated with tangles (Zhukareva *et al.*, 2004). With respect to diffuse plaques, their formation presumably originates in attachment of oligomers to synapses. A threshold for memory loss would be determined by the degree to which synapses can remain unoccupied and functional, giving a cellular basis for the concept of cognitive reserve. Extent of memory loss would reflect the fractional occupancy of toxin receptors at particular synapses as well as fractional occupancy of all at-risk synapses. Fluctuations in occupancy, whether due to ligand disassociation or receptor replacement or synaptic turnover, would account for day-to-day fluctuations in cognitive performance, common in early AD. Finally, given the key role played by diffusible subfibrillar toxins, the new cascade explains why AD correlates so poorly with amyloid plaque burden.

Like the original amyloid cascade hypothesis, the emended cascade presumably will continue to evolve. Three issues for the future will be considered briefly in the remaining sections: (1) what is the relationship between oligomers and the process of fibrillogenesis? (2) Do toxic oligomers of A β provide precedent for mechanisms common to multiple fibrillogenic proteins? (3) Does the new amyloid cascade provide a rational basis for effective AD therapeutics and diagnostics?

12. Mechanisms of A β Oligomerization and Fibrillogenesis

In multiple milieus (AD brain; mouse models; *in vitro*), A β 1-42 assembles to form soluble oligomers as well as insoluble amyloid fibrils. How these disparate outcomes are achieved is not fully understood, and whether smaller implies precursor to the larger remains speculative. At issue is whether oligomers and fibrils form via alternative mechanisms. Are there distinct pathways for assembly, or are oligomers simply metastable intermediates that eventually cobble together to create fibrils?

Insight into precursor-product relationships comes from early studies of A β fibrillogenesis. Two groups (Harper *et al.*, 1997; Walsh *et al.*, 1997) independently established the existence of sub-

fibrillar intermediates called protofibrils (PFs), which are structurally distinct from the small oligomers discussed so far. Protofibrils are linear rather than globular, they extend in length up to 400 nm, and they develop masses up to one million daltons. Kinetic experiments indicate that protofibrils are the immediate precursors to full-fledged fibrils (Walsh *et al.*, 1997). The pathogenic protofibril-to-fibril pathway, which includes the concept of a critical concentration for monomer to drive assembly, in many respects resembles the physiological process of cytoskeletal protein assembly.

Initially, protofibrils were thought to be relevant only as structural intermediates, existing transiently en route to production of toxic amyloid fibrils. Evidence now indicates that PFs themselves are neurologically active. In neuronal cultures, PFs trigger action potentials (Hartley *et al.*, 1999), and they ultimately induce neuronal death (Walsh *et al.*, 1999). It has not been reported whether PFs block synaptic plasticity. Unlike small oligomers, however, PFs appear to attach nonselectively to neurons, broadly coating entire cell surfaces (Hartley *et al.*, 1999). The presence of PFs in AD brain has not been established, although they reportedly occur in cerebral spinal fluid of AD patients (Pitschke *et al.*, 1998). Whether or not PFs are clinically relevant, the current molecular and cellular data indicate that A β self-assembles into two different subfibrillar A β species, each with cytotoxic activity.

PFs generate fibrils, but do globular oligomers generate PFs? If so, it would place oligomers on the pathway to fibrillogenesis. Under some conditions oligomers and PFs are evident in the same preparation. Molecular imaging by AFM, for example, shows the coexistence of oligomer-sized particles and PFs; PFs, moreover, exhibit a “bead-on-a-string” morphology, suggesting arrays of oligomers that have associated (Walsh *et al.*, 1999; Chromy *et al.*, 2003) (**Figure 4-4; see color insert**). Protofibrils subjected to denaturing gel electrophoresis comprise monomers and SDS-stable oligomers. Support for a precursor–product relationship also comes from a new approach to oligomerization and fibrillogenesis using photo-induced crosslinking, which stabilizes all species for subsequent electrophoresis (Bitan *et al.*, 2003a). A β 42 generates three classes of SDS-stable oligomers, and the largest, which comprise 12 mers and 18 mers, appear to give rise to molecules the size of PFs, detected by light scattering. The kinetics, however, have not yet been verified. Significantly, A β 40, which is much more abundant in brain than A β 42 and less germane to AD, does not give rise to the larger oligomers. Bitan *et al.* suggest that differences in ability to populate the higher order oligomer states would account for the difference in biological activity between A β 40 and A β 42.

Immunoanalysis of oligomers and PFs suggests that if a precursor–product relationship does exist, it is less simple than beads-on-a-string. Antibodies have been generated that recognize oligomers but not PFs or fibrils (Lambert *et al.*, 2001; Chromy *et al.*, 2003); this implies that if oligomers were to generate PFs, the antibody-sensitive domains either become masked or undergo conformation changes. Precedent for this idea is found in studies of fibrillogenesis of a multiple myeloma immunoglobulin light chain, in which fibrils from off-pathway oligomers are generated only after structural rearrangement of metastable oligomers to a disordered species more prone to fibrillogenesis (Souillac *et al.*, 2003).

Relevant to assembly *in vivo*, conditions have been established that permit oligomers to form *in vitro* at low nanomolar A β concentrations (Yu *et al.*, 2002). Even when prepared at micromolar A β , oligomers can be maintained for days without formation of protofibrils (Chromy *et al.*, 2003), in harmony with the accumulation of oligomers as molecular entities in AD brain. The basis for the relative stability of oligomers is unknown, although it may derive from the unusual charge structure of A β , with its two lengthy hydrophilic/hydrophobic domains. The hydrophobic C-terminal is clearly influential given the much greater stability of A β 42 oligomers over those of A β 40 (Stine *et al.*, 2003). Photo-induced crosslinking experiments indicate, moreover, that alanine at the 42 position is essential for generating quantum-like jumps in formation of 12 mers and 18 mers (Bitan *et al.*, 2003b). Covalent changes also may be relevant. For example, acceleration of oligomerization has been found to be cata-

lyzed by prostaglandin H2, suggested to link pathogenic oligomerization to cyclooxygenase (Boutaud *et al.*, 2002), a possible target for AD therapeutics. Small oligomers also are susceptible to interpeptide crosslinking through metal ion-catalyzed oxidative reactions (Atwood *et al.*, 2004; Butterfield and Bush, 2004), a finding that has engendered development of copper-selective chelators as possible therapeutic drugs (Cherny *et al.*, 2001). Oxidative crosslinking of oligomers may not be required for their SDS stability, because replacement of the redox-relevant methionine at position 35 does not preclude oligomerization (Klein *et al.*, 2004), but crosslinking could potentially influence oligomer conformation. Evidence that oligomers of the same size can develop different structures comes from immunoblot experiments with conformation-sensitive antibodies (Chromy *et al.*, 2003).

States of initial conformation are likely to be especially relevant to the outcome of self-assembly. Monomeric A β , as all proteins, will exist at least transiently in multiple conformation states (Ferreira and De Felice, 2001). Hypothetically, given sufficiently deep energy wells, alternative monomer conformations could be sufficiently long-lived to generate alternative structural outcomes for self-assembly. One metastable conformer, for example, could generate oligomers while another generates protofibrils and fibrils. Conformational shifts may account for the chaperone-like action of ApoJ (clusterin) in promoting oligomer accumulation while retarding fibril formation. Well-recognized inconsistencies in experiments with A β suggest the peptide indeed has significant conformational sensitivity to micromilieu. Because experimental conditions influence A β and the nature of its self-assembly, considerable potential exists for *in vitro* artifacts. This underscores the value of verifying that experimentally produced oligomers mimic the properties of AD brain-derived oligomers, as recently reported (Gong *et al.*, 2003).

Unfortunately, while mechanistic understanding of oligomer and fibril assembly *in vitro* is limited, even less is known regarding assembly *in vivo*. The list of unanswered or partially answered questions is lengthy. What is the origin of oligomers in AD—do they form inside cells or out? Is oligomerization *in vivo* favored by mis-folding, or is it driven simply by mass action, because A β has such a strong propensity for self-assembly? Does synaptic binding imply oligomers have physiological functions? A β , after all, is a product of remarkably complex processing that involves multiple cell compartments, proteases, trafficking, and signal-dependent regulation. Perhaps oligomers normally control positive synaptic feedback or influence synaptic turnover? Why is oligomer accumulation age dependent? Why, for example, do oligomers not form during development or in mature adults despite the same levels of APP? Are there chaperones present to prevent oligomer formation, perhaps part of the regulatory process? Do these chaperones become less active in AD? What is the relationship between the synaptic binding of oligomers and the formation of amyloid fibrils? Do oligomers at synapses act as seeds for fibril formation? Might binding to toxin receptors alter the conformation of oligomers, engendering formation of bead-on-a-string protofibrils and the subsequent generation of fibrils? Would elimination of oligomers keep new fibrils from forming and accelerate the removal of old ones?

13. Pathogenic A β Oligomers—First of Many? All Proteins Likely Have the Capacity to Oligomerize

For AD, the amyloid cascade has been a fertile and adaptive hypothesis, evolving both with respect to toxin structure and the underlying cellular mechanisms. A pertinent question is whether the concepts developed for A β might apply to other diseases. Might other fibrillogenic proteins, which have been linked to a multitude of diseases, similarly produce subfibrillar pathogenic assemblies? For at least several cases, the answer appears to be “yes,” although the evidence is far from conclusive.

Data from Parkinson's disease (PD), the second most common neurodegenerative disorder in aging, are the most developed. Three mutations have been found that implicate the fibrillogenic protein α -synuclein in loss of dopamine-producing neurons (Caughey and Lansbury, 2003). In PD pathology, fibrils of α -synuclein accumulate intracellularly, in structures called Lewy bodies (Norris *et al.*, 2004), while overexpression of α -synuclein in cultured cells causes death of dopaminergic neurons (Xu *et al.*, 2002). *In vitro* studies of fibrillogenesis by Conway, Lansbury, and colleagues (Caughey and Lansbury, 2003) show sequential appearance of structures analogous to those described for A β : small globular structures, bead-on-a-string protofibrils, and large fibrils. Self-association is promoted by two PD-coupled point mutations in α -synuclein. However, although both mutations accelerate assembly of the globular intermediates, one of the mutations actually inhibits fibrillogenesis (Conway *et al.*, 2000), which led to the hypothesis that subfibrillar species constitute the pathogenic agents. Consistent with this idea, strains of tg mice have been developed that lose dopaminergic synapses and develop abnormalities in motor behavior even though their α -synuclein inclusions are nonfibrillar (Masliah *et al.*, 2000). Similarly, lentiviral transfection has produced rats whose α -synuclein inclusions are nonfibrillar and that suffer dopaminergic neuron death (Lo Bianco *et al.*, 2002); in contrast, transfected rats with fibrillar inclusions show no cell death. A corresponding situation is evident in the human brain: individuals without disease symptoms often present fibril-containing Lewy bodies. These cases (asymptomatic incidental Lewy body disease) are 10 times more frequent than PD (Goldberg and Lansbury, 2000). It has been proposed that incidental Lewy body disease may result when α -synuclein rapidly produces fibrils in a manner that excludes accumulation of pathogenic, subfibrillar species (Caughey and Lansbury, 2003). Similarly, with respect to A β , it is not uncommon for individuals to present high levels of amyloid plaques without showing symptoms of Alzheimer's disease (Katzman *et al.*, 1988). Mechanistically, the subfibrillar forms of α -synuclein attach and insert into membranes with higher affinity than fibrils (Ding *et al.*, 2002), potentially creating cytotoxic pores. Pore-like structures generated *in vitro* by α -synuclein are evident by atomic force microscopy, and PD mutations foster production of the pore-like structures (Volles and Lansbury, 2002). More recently, it also has been reported that α -synuclein oligomers, as well as fibrils, can bind to proteasomes and inhibit protease activity (Lindersson *et al.*, 2004).

Unlike PD and AD, the human spongiform encephalopathies are rare, but interest is very high given their ability to be transmitted between individuals and species (e.g., "mad cow disease"). Research largely has focused on verifying that disease transmission is based, surprisingly, on proteins (prions) rather than nucleic acids. Prions in their abnormal, disease-state conformation are fibrillogenic, and they accumulate in diseased brain as ordered aggregates and amyloid plaques (Fournier and Grigoriev, 2001). In disease transmission, pathogenic prions convert normal cellular prions to the abnormal conformation. Conversion may involve disease-state prions in aggregated form, with a stabilizing energy-well provided by aggregation. It has been noted that small oligomers of prions also can be formed, and that their assembly is off-pathway with respect to fibrillogenesis (Baskakov *et al.*, 2002). Whether the oligomers are neurotoxic is unknown, although a recent study of the yeast prion system suggests that small oligomeric species are important for transmission (Narayanan *et al.*, 2003). At present, the relationship between states of prion aggregation, the structures required for transmission, and those essential for neurotoxicity all remain to be clarified.

A brief overview of other studies further indicates that production of cytotoxic, subfibrillar oligomers is an aspect of pathogenesis common to multiple diseases of fibrillogenic proteins. These oligomers are implicated not only in neurological diseases but in various other organ failures. (1) *ADan* and *ABri*. Mutations in the *BRI* gene cause AD-like dementia with additional neurological dysfunctions. The mutations promote release of two fibrillogenic peptides, *ADan* and *ABri*, in the brain (Austen *et al.*, 2002). The peptides are found, respectively, in familial Danish dementia and familial British dementia. Culture experiments show toxicity associated with formation of SDS-

stable, nonfibrillar, low-molecular-mass oligomers. The small oligomers are more potent in inducing neuronal apoptosis than PFs or fibrils (El Agnaf *et al.*, 2001). Recently, deposits of nonfibrillar ADan aggregates have been found in the brain (Gibson *et al.*, 2004). (2) *Transthyretin*. Mutations in transthyretin (TTr) produce amyloidoses associated with familial amyloid polyneuropathy and with familial amyloid cardiomyopathy. Cell culture experiments indicate that small soluble aggregates are cytotoxic species, with no toxicity found associated with TTR amyloid fibrils or soluble aggregates greater than 100 kDa (Reixach *et al.*, 2004). (3) *IAPP*. Type II diabetes is associated with islet amyloid polypeptide (IAPP), a fibrillogenic protein cosecreted with insulin. Insulin resistance leads to excessive release of IAPP. Results with a murine model of type 2 diabetes indicate that the formation of islet amyloid, rather than the amyloid per se, is related to increased β -cell apoptosis. These findings, suggesting that pathogenesis, might be coupled to subfibrillar IAPP oligomers rather than islet amyloid (Butler *et al.*, 2003), are recently substantiated by findings that IAPP can make toxic oligomers that induce apoptosis in replicating β -cells (Butler *et al.*, 2004). IAPP toxins in cell culture are aggregated but not necessarily fibrillar. Protofibrillar species have been observed that exhibit pore-like channel activity, like the PFs of α -synuclein (Anguiano *et al.*, 2002). (4) *Immunoglobulin light chain*. Immunoglobulin light-chain amyloidosis is associated with organ damage. As with IAPP, clinical findings suggest the process of amyloid fibril formation (i.e., putatively, oligomer formation) itself exerts toxic effects, independently of the amount of amyloid deposited (Bellotti *et al.*, 2000). As mentioned earlier, some light chains generate off-pathway oligomers that must become disordered before fibrils can form (Souillac *et al.*, 2003). By *in situ* AFM, immunoglobulin light chain structures can be seen that are similar to the annular or taurus-shaped morphologies found to be toxic with α -synuclein PFs (Zhu *et al.*, 2004). (5) *Beta-2-microglobulin*. Serum levels of fibrillogenic β -2 microglobulin rise during renal failure, and dialysis-associated amyloidosis shows deposits in joints, bones, and organs. Evidence from single-channel conductance experiments suggest oligomers of β -2-microglobulin form channel structures, again supporting the oligomer-pore hypothesis for pathogenesis (Hirakura and Kagan, 2001).

Normal soluble proteins, not just those that are disease associated, have a capacity for fibrillogenesis, and they generate fibrils that are indistinguishable from those in pathological amyloid deposits (Dobson, 1999). Like their disease-associated counterparts, the normal proteins also produce subfibrillar aggregates. These are sufficiently stable to be isolated, and in two recently studied examples, the subfibrillar species, but not the full-fledged fibrils, were found to be cytotoxic (Bucciantini *et al.*, 2002). Bucciantini *et al.* have suggested that inherent cytotoxicity of subfibrillar aggregates might provide a common mechanism for protein misfolding diseases. They further proposed that toxicity derives from the common structural nature of the subfibrillar aggregates, not their specific amino acid sequences. Supporting this, certain antibodies generated against subfibrillar aggregates of A β recognize disparate oligomer species regardless of monomer sequence, suggestive of a common conformation-dependent structure (Kayed *et al.*, 2003). On the other hand, the highly specific targeting of particular synapses by ADDLs would seem less consistent with completely generic mechanisms of pathogenesis. Conceivably, different aspects of oligomer cytotoxicity may derive from different structural determinants.

14. Therapeutics and Diagnostics—New Strategies

Insights gleaned from examining A β oligomers as a case study extend to therapeutics and diagnostics. In a remarkable new strategy that shows increasing promise, AD researchers have been seeking to develop active vaccines and therapeutic antibodies that can neutralize A β -derived neurotoxins. This strategy derives from the benchmark discovery by Schenk *et al.* (1999) that amyloid

plaques can be removed from brains of tg mice models by vaccination with preparations of A β . These preparations prior to vaccination are aged to promote formation of fibrils, although such preparations are heterogeneous and also contain subfibrillar A β species (Chromy *et al.*, 2003), which themselves are highly immunogenic (Lambert *et al.*, 2001). Two conclusions from the Schenk study were almost completely unexpected: (1) fibrillar plaque molecules, which are resistant to many disrupting agents, can be eliminated biologically; and (2) therapeutic levels of antibodies can actually reach the brain, typically considered to be immuno-privileged. Cognitive benefits of active vaccination subsequently were reported by two other groups in studies that suggested the relevant targets might be subfibrillar (Janus *et al.*, 2000; Morgan *et al.*, 2000).

Clinical trials ensued shortly after the successful experiments with mouse vaccines, and, although these trials failed, they nonetheless provided a significant proof of concept. Vaccine trials with AD patients were terminated because of high incidence of brain inflammation and occurrence of patient death (Ferrer *et al.*, 2004). It is uncertain whether inflammation derived from aberrant T-cell activation by the vaccine or from localized responses induced by antibodies attached to plaques. However, despite its great failings, the AD vaccine trial provided a truly exciting result: in individuals who mounted a robust immune response, the progression of Alzheimer's disease over a 12-month period was stopped in its tracks (Hock *et al.*, 2003).

The potential to capture the vaccine's success while avoiding its failings has motivated vigorous efforts to develop therapeutic monoclonal antibodies by Elan, Lilly, and Merck. Unlike active vaccines, such antibodies would be unaffected by problems such as T-cell-mediated inflammation or the impaired immune response of elderly individuals. Precedent for success is evident in the memory-recovery experiments with tg mice models (Dodart *et al.*, 2002; Kotilinek *et al.*, 2002), which also indicated that elimination of subfibrillar species is more relevant than elimination of fibrils. Antibodies already have been described that neutralize oligomers without binding A β monomers or fibrils (Chromy *et al.*, 2003). Such specificity would lower antibody dosage by avoiding monomers and further reduce risks of inflammation by not binding plaques. Soluble oligomers thus appear to present an optimal target for therapeutic antibodies.

Oligomers also may be important targets for therapeutic drugs. Assembly of oligomers from monomer can be blocked *in vitro* by small organic molecules (De Felice *et al.*, 2004), some with efficacy at doses under 100 nanomolar (Wang *et al.*, 2004b). Certain natural products also are effective. Extracts of *Ginkgo biloba*, anecdotally considered to have cognitive benefits, are surprisingly effective at blocking ADDL assembly (Yao *et al.*, 2001; Chromy *et al.*, 2003). Also, considered earlier, copper chelators and cyclooxygenase inhibitors may be useful in blocking aspects of oligomerization (Cherny *et al.*, 2001; Boutaud *et al.*, 2002). ADDL toxin receptors, as yet undiscovered, may provide targets for development of ADDL antagonists, while intervention in pertinent signal transduction pathways also might overcome ADDL synaptotoxicity. Molecules such as memantine or nonsteroidal anti-inflammatory drugs hypothetically might act at such levels.

Development of AD therapeutics, whether vaccines or drugs, will almost certainly be accelerated by invention of a valid molecular diagnostic. Although promise has emerged for detection of amyloid plaques by imaging (Klunk *et al.*, 1994, 2003), it would be valuable to diagnose the disease at earlier stages. Whether oligomers might provide a biomarker is not yet clear. Striking differences exist between oligomer levels in AD and normal brain extracts (Gong *et al.*, 2003), but no data are available concerning early stages of the disease or mild cognitive impairment (MCI). An appealing property of oligomers, however, is their solubility, which suggests they could diffuse from brain into cerebral spinal fluid or blood. Detection of extremely low levels of peripheral oligomers now appears feasible given advances in nanotechnology, which provide limits of detection that are orders of magnitude better than currently possible (Haes *et al.*, 2004). One method, which uses a combination of immunomagnetic beads and nanoparticle-based polymerase chain reaction (PCR) (Nam *et al.*, 2003),

can detect as few as 50 molecules of ADDLs; applied to 30 patients, the method has found ADDLs in cerebral spinal fluid at a level 1000% of that in age-matched controls (Georganopolou *et al.*, 2005). These early results suggest that oligomer-based diagnostics might become possible in the near future.

Ultrasensitive diagnostics developed for AD potentially can be adapted to other diseases of fibrillogenic proteins such as human spongiform encephalopathy. Similarly with respect to therapeutics, successful precedents with experimental antibodies against A β oligomers suggest adaptation to other diseases merits investigation. Ultimately, lessons learned from a case study of AD's subfibrillar toxins, besides providing new ways to think about fibrillogenic proteins and disease mechanisms, may establish a broad platform for new approaches to therapeutics and diagnostics.

15. Abbreviations

A β	Amyloid β peptide
AD	Alzheimer's disease
ADDL	A β -derived diffusible ligands
AFM	Atomic force microscopy
APP	Amyloid precursor protein
CNS	Central neural system
LTD	Long-term depression
LTP	Long-term potentiation
PD	Parkinson's disease
PFs	Protofibrils
tg mice	Transgenic mice
TTr	Transthyretin

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Potential Conflicts

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References

- Aksenov, M.Y., Aksenova, M.V., Butterfield, D.A., Hensley, K., Vigo-Pelfrey, C., and Carney, J.M. (1996). Glutamine synthetase-induced enhancement of beta-amyloid peptide A beta (1–40) neurotoxicity accompanied by abrogation of fibril formation and A beta fragmentation. *J. Neurochem.* 66:2050–2056.

- Allison, D.W., Chervin, A.S., Gelfand, V.I., and Craig, A.M. (2000). Postsynaptic scaffolds of excitatory and inhibitory synapses in hippocampal neurons: maintenance of core components independent of actin filaments and microtubules. *J. Neurosci.* 20:4545–4554.
- Alzheimer, A. (1907). Über eine eigenartige Erkrankung der Hirnrinde. *Allge. Z. Psychiatr. Phych.-Gerich. Med. (Berl.)* 64:146–148.
- Anguiano, M., Nowak, R.J., and Lansbury, P.T., Jr. (2002). Protofibrillar islet amyloid polypeptide permeabilizes synthetic vesicles by a pore-like mechanism that may be relevant to type II diabetes. *Biochemistry* 41:11338–11343.
- Arispe, N., Rojas, E., and Pollard, H.B. (1993). Alzheimer disease amyloid beta protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminum. *Proc. Nat. Acad. Sci. USA* 90:567–571.
- Arispe, N., Pollard, H.B., and Rojas, E. (1994). beta-Amyloid Ca(2+)-channel hypothesis for neuronal death in Alzheimer disease. *Mol. Cell Biochem.* 140:119–125.
- Atwood, C.S., Perry, G., Zeng, H., Kato, Y., Jones, W.D., Ling, K.Q., Huang, X., Moir, R.D., Wang, D., Sayre, L.M., Smith, M.A., Chen, S.G., and Bush, A.I. (2004). Copper mediates dityrosine cross-linking of Alzheimer's amyloid-beta. *Biochemistry* 43:560–568.
- Austen, B., el Agnaf, O., Nagala, S., Patel, B., Gunasekera, N., Lee, M., and Lelyveld, V. (2002). Properties of neurotoxic peptides related to the BRI gene. *Biochem. Soc. Trans.* 30:557–559.
- Baskakov, I.V., Legname, G., Baldwin, M.A., Prusiner, S.B., and Cohen, F.E. (2002). Pathway complexity of prion protein assembly into amyloid. *J. Biol. Chem.* 277:21140–21148.
- Bellotti, V., Mangione, P., and Merlini, G. (2000). Review: immunoglobulin light chain amyloidosis—the archetype of structural and pathogenic variability. *J. Struct. Biol.* 130:280–289.
- Bennett, M.R. (2000). The concept of long term potentiation of transmission at synapses. *Prog. Neurobiol.* 60:109–137.
- Bitan, G., Kirkitadze, M.D., Lomakin, A., Vollers, S.S., Benedek, G.B., and Teplow, D.B. (2003a). Amyloid beta-protein (A β) assembly: A β 40 and A β 42 oligomerize through distinct pathways. *Proc. Natl. Acad. Sci. USA* 100:330–335.
- Bitan, G., Vollers, S.S., and Teplow, D.B. (2003b). Elucidation of primary structure elements controlling early amyloid beta-protein oligomerization. *J. Biol. Chem.* 278:34882–34889.
- Borchelt, D.R., Thinakaran, G., Eckman, C.B., Lee, M.K., Davenport, F., Ratovitsky, T., Prada, C.M., Kim, G., Seekins, S., Yager, D., Slunt, H.H., Wang, R., Seeger, M., Levey, A.I., Gandy, S.E., Copeland, N.G., Jenkins, N.A., Price, D.L., Younkin, S.G., and Sisodia, S.S. (1996). Familial Alzheimer's disease-linked presenilin 1 variants elevate A β 42/40 ratio in vitro and in vivo. *Neuron* 17:1005–1013.
- Boutaud, O., Ou, J.J., Chaurand, P., Caprioli, R.M., Montine, T.J., and Oates, J.A. (2002). Prostaglandin H2 (PGH2) accelerates formation of amyloid beta1-42 oligomers. *J. Neurochem.* 82:1003–1006.
- Braak, H., and Braak, E. (1991). Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol. (Berl.)* 82:239–259.
- Bucciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C.M., and Stefani, M. (2002). Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature* 416:507–511.
- Burdick, D., Soreghan, B., Kwon, M., Kosmoski, J., Knauer, M., Henschen, A., Yates, J., Cotman, C., and Glabe, C. (1992). Assembly and aggregation properties of synthetic Alzheimer's A4/beta amyloid peptide analogs. *J. Biol. Chem.* 267:546–554.
- Busciglio, J., Lorenzo, A., Yeh, J., and Yankner, B.A. (1995). beta-Amyloid fibrils induce tau phosphorylation and loss of microtubule binding. *Neuron* 14:879–888.
- Butler, A.E., Janson, J., Soeller, W.C., and Butler, P.C. (2003). Increased beta-cell apoptosis prevents adaptive increase in beta-cell mass in mouse model of type 2 diabetes: Evidence for role of islet amyloid formation rather than direct action of amyloid. *Diabetes* 52:2304–2314.
- Butler, A.E., Jang, J., Gurlo, T., Carty, M.D., Soeller, W.C., and Butler, P.C. (2004). Diabetes due to a progressive defect in beta-cell mass in rats transgenic for human islet amyloid polypeptide (HIP Rat): a new model for type 2 diabetes. *Diabetes* 53:1509–1516.
- Butterfield, D.A., and Bush, A.I. (2004). Alzheimer's amyloid beta-peptide (1–42): involvement of methionine residue 35 in the oxidative stress and neurotoxicity properties of this peptide. *Neurobiol. Aging* 25:563–568.
- Buttini, M., Yu, G.Q., Shockley, K., Huang, Y., Jones, B., Masliah, E., Mallory, M., Yeo, T., Longo, F.M., and Mucke, L. (2002). Modulation of Alzheimer-like synaptic and cholinergic deficits in transgenic mice by human apolipoprotein E depends on isoform, aging, and overexpression of amyloid beta peptides but not on plaque formation. *J. Neurosci.* 22:10539–10548.

- Caughey, B., and Lansbury, P.T., Jr. (2003). Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders. *Annu. Rev. Neurosci.* 26:267–298.
- Chang, L., Bakchos, L., Wang, Z., Venton, D.L., and Klein, W.L. (2003). Femtomole immunodetection of synthetic and endogenous Amyloid- β oligomers and its application to Alzheimer's Disease drug candidate screening. *J. Mol. Neurosci.* 20:305–313.
- Chartier-Harlin, M.C., Crawford, F., Houlden, H., Warren, A., Hughes, D., Fidani, L., Goate, A., Rossor, M., Roques, P., and Hardy, J. (1991). Early-onset Alzheimer's disease caused by mutations at codon 717 of the beta-amyloid precursor protein gene. *Nature* 353:844–846.
- Cherny, R.A., Atwood, C.S., Xilinas, M.E., Gray, D.N., Jones, W.D., McLean, C.A., Barnham, K.J., Volitakis, I., Fraser, F.W., Kim, Y., Huang, X., Goldstein, L.E., Moir, R.D., Lim, J.T., Beyreuther, K., Zheng, H., Tanzi, R.E., Masters, C.L., and Bush, A.I. (2001). Treatment with a copper–zinc chelator markedly and rapidly inhibits beta-amyloid accumulation in Alzheimer's disease transgenic mice. *Neuron* 30:665–676.
- Chin, J., Palop, J.J., Yu, G.Q., Kojima, N., Masliah, E., and Mucke, L. (2004). Fyn kinase modulates synaptotoxicity, but not aberrant sprouting, in human amyloid precursor protein transgenic mice. *J. Neurosci.* 24:4692–4697.
- Chromy, B.A., Nowak, R.J., Lambert, M.P., Viola, K.L., Chang, L., Velasco, P.T., Jones, B.W., Fernandez, S.J., Lacor, P.N., Horowitz, P., Finch, C.E., Krafft, G.A., and Klein, W.L. (2003). Self-assembly of A β (1–42) into globular neurotoxins. *Biochemistry* 42:12749–12760.
- Constantine-Paton, M., and Cline, H.T. (1998). LTP and activity-dependent synaptogenesis: the more alike they are, the more different they become. *Curr. Opin. Neurobiol.* 8:139–148.
- Conway, K.A., Lee, S.J., Rochet, J.C., Ding, T.T., Williamson, R.E., and Lansbury, P.T., Jr. (2000). Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy. *Proc. Natl. Acad. Sci. USA* 97:571–576.
- Coyle, J.T. (1987). Alzheimer's disease. In: Adelman, G. (Eds.), *Encyclopedia of neuroscience*, Vol.1. Boston: Birkhauser, pp. 29–31.
- da Cruz e Silva, E.F., and da Cruz e Silva, O.A. (2003). Protein phosphorylation and APP metabolism. *Neurochem. Res.* 28:1553–1561.
- De Felice, F.G., Vieira, M.N.N., Saraiva, L.M., Figueroa-Villar, J.D., Garcia-Abreu, J., Liu, R., Chang, L., Klein, W.L., and Ferreira, S.T. (2004). Targeting the neurotoxic species in Alzheimer's disease: small molecule inhibitors of the formation of soluble oligomers and fibrils of the β -amyloid peptide. *FASEB J.* 18:1366–1372.
- Ding, T.T., Lee, S.J., Rochet, J.C., and Lansbury, P.T., Jr. (2002). Annular alpha-synuclein protofibrils are produced when spherical protofibrils are incubated in solution or bound to brain-derived membranes. *Biochemistry* 41:10209–10217.
- Dobson, C.M. (1999). Protein misfolding, evolution and disease. *Trends Biochem. Sci.* 24:329–332.
- Dodart, J.C., Bales, K.R., Gannon, K.S., Greene, S.J., DeMattos, R.B., Mathis, C., DeLong, C.A., Wu, S., Wu, X., Holtzman, D.M., and Paul, S.M. (2002). Immunization reverses memory deficits without reducing brain A β burden in Alzheimer's disease model. *Nat. Neurosci.* 5:452–457.
- El Agnaf, O.M., Nagala, S., Patel, B.P., and Austen, B.M. (2001). Non-fibrillar oligomeric species of the amyloid ABri peptide, implicated in familial British dementia, are more potent at inducing apoptotic cell death than protofibrils or mature fibrils. *J. Mol. Biol.* 310:157–168.
- Ferreira, S.T., and De Felice, F.G. (2001). PABMB Lecture. Protein dynamics, folding and misfolding: from basic physical chemistry to human conformational diseases. *FEBS Lett.* 498:129–134.
- Ferrer, I., Boada, R.M., Sanchez Guerra, M.L., Rey, M.J., and Costa-Jussa, F. (2004). Neuropathology and pathogenesis of encephalitis following amyloid-beta immunization in Alzheimer's disease. *Brain Pathol.* 14:11–20.
- Fiala, J.C., Spacek, J., and Harris, K.M. (2002). Dendritic spine pathology: cause or consequence of neurological disorders? *Brain Res. Brain Res. Rev.* 39:29–54.
- Fournier, J.G., and Grigoriev, B. (2001). Prion diseases: contribution of high-resolution immunomorphology. *J. Cell Mol. Med.* 5:367–377.
- Gardoni, F., Bellone, C., Viviani, B., Marinovich, M., Meli, E., Pellegrini-Giampietro, D.E., Cattabeni, F., and Di Luca, M. (2002). Lack of PSD-95 drives hippocampal neuronal cell death through activation of an alpha CaMKII transduction pathway. *Eur. J. Neurosci.* 16:777–786.
- Georganopoulou, D.G., Chang, L., Klein, W.L., and Mirkin, C.A. (2005). Nanoparticle-based detection of a soluble pathogenic biomarker for Alzheimer's disease. *Proc. Natl. Acad. Sci.* 102:2273–2276.
- Gibson, G., Gunasekera, N., Lee, M., Lelyveld, V., El Agnaf, O.M., Wright, A., and Austen, B. (2004). Oligomerization and neurotoxicity of the amyloid ADan peptide implicated in familial Danish dementia. *J. Neurochem.* 88: 281–290.
- Glenner, G.G., and Wong, C.W. (1984). Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* 120:885–890.

- Goate, A., Chartier-Harlin, M.C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., and James, L. (1991). Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease [see comments]. *Nature* 349:704–706.
- Goldberg, M.S., and Lansbury, P.T., Jr. (2000). Is there a cause-and-effect relationship between alpha-synuclein fibrilization and Parkinson's disease? *Nat. Cell Biol.* 2:E115–E119.
- Gong, Y., Chang, L., Viola, K.L., Lacor, P.N., Lambert, M.P., Finch, C.E., Krafft, G.A., and Klein, W.L. (2003). Alzheimer's disease-affected brain: presence of oligomeric A β ligands (ADDLs) suggests a molecular basis for reversible memory loss. *Proc. Natl. Acad. Sci. USA* 100:10417–10422.
- Guzowski, J.F. (2002). Insights into immediate-early gene function in hippocampal memory consolidation using antisense oligonucleotide and fluorescent imaging approaches. *Hippocampus* 12:86–104.
- Haes, A.E., Hall, W.P., Chang, L., Klein, W.L., and Van Duyne, R.P. (2004). A localized surface plasmon resonance biosensor: first steps toward an assay for Alzheimer's disease. *Nano Lett.* 4:1029–1034.
- Hardy, J.A., and Higgins, G.A. (1992). Alzheimer's disease: the amyloid cascade hypothesis. *Science* 256:184–185.
- Hardy, J., and Selkoe, D.J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297:353–356.
- Harper, J.D., Wong, S.S., Lieber, C.M., and Lansbury, P.T., Jr. (1997). Observation of metastable abeta amyloid protofibrils by atomic force microscopy. *Chem. Biol.* 4:119–125.
- Hartley, D.M., Walsh, D.M., Ye, C.P., Diehl, T., Vazquez, S., Vassilev, P.M., Teplow, D.B., and Selkoe, D.J. (1999). Protofibrillar intermediates of amyloid beta-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. *J. Neurosci.* 19:8876–8884.
- Hebert, L.E., Scherr, P.A., Bienias, J.L., Bennett, D.A., and Evans, D.A. (2003). Alzheimer disease in the US population: prevalence estimates using the 2000 census. *Arch. Neurol.* 60:1119–1122.
- Hensley, K., Butterfield, D.A., Mattson, M., Aksenova, M., Harris, M., Wu, J.F., and Carney, J. (1995). A model for beta-amyloid aggregation and neurotoxicity based on the free radical generating capacity of the peptide: implications of "molecular shrapnel" for Alzheimer's disease. *Proc. West. Pharmacol. Soc.* 38:113–120.
- Hilbich, C., Kisters-Woike, B., Reed, J., Masters, C.L., and Beyreuther, K. (1991). Aggregation and secondary structure of synthetic amyloid beta A4 peptides of Alzheimer's disease. *J. Mol. Biol.* 218:149–163.
- Hirakura, Y., and Kagan, B.L. (2001). Pore formation by beta-2-microglobulin: a mechanism for the pathogenesis of dialysis associated amyloidosis. *Amyloid* 8:94–100.
- Hock, C., Konietzko, U., Streffer, J.R., Tracy, J., Signorell, A., Muller-Tillmanns, B., Lemke, U., Henke, K., Moritz, E., Garcia, E., Wollmer, M.A., Uebrecht, D., de Quervain, D.J., Hofmann, M., Maddalena, A., Papassotiropoulos, A., and Nitsch, R.M. (2003). Antibodies against beta-amyloid slow cognitive decline in Alzheimer's disease. *Neuron* 38:547–554.
- Hsia, A.Y., Masliah, E., McConlogue, L., Yu, G.Q., Tatsuno, G., Hu, K., Kholodenko, D., Malenka, R.C., Nicoll, R.A., and Mucke, L. (1999). Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. *Proc. Natl. Acad. Sci. USA* 96:3228–3233.
- Janus, C., Pearson, J., McLaurin, J., Matthews, P.M., Jiang, Y., Schmidt, S.D., Chishti, M.A., Horne, P., Heslin, D., French, J., Mount, H.T.J., Nixon, R.A., Mercken, M., Bergeron, C., Fraser, P.E., St. George-Hyslop, P., and Westaway, D. (2000). ABeta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease. *Nature* 408:979–982.
- Katzman, R., Terry, R., DeTeresa, R., Brown, T., Davies, P., Fuld, P., Renbing, X., and Peck, A. (1988). Clinical, pathological, and neurochemical changes in dementia: a subgroup with preserved mental status and numerous neocortical plaques. *Ann. Neurol.* 23:138–144.
- Kayed, R., Head, E., Thompson, J.L., McIntire, T.M., Milton, S.C., Cotman, C.W., and Glabe, C.G. (2003). Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science* 300:486–489.
- Kelly, M.P., and Deadwyler, S.A. (2003). Experience-dependent regulation of the immediate-early gene arc differs across brain regions. *J. Neurosci.* 23:6443–6451.
- Kim, H.J., Chae, S.C., Lee, D.K., Chromy, B., Lee, S.C., Park, Y.C., Klein, W.L., Krafft, G.A., and Hong, S.T. (2003). Selective neuronal degeneration induced by soluble oligomeric amyloid beta protein. *FASEB J.* 17:118–120.
- Klein, W., Krafft, G., and Finch, C.E. (2001). Targeting small Amyloid-beta oligomers: the solution to an Alzheimer's disease conundrum? *Trends Neurosci.* 24:219–224.
- Klein, W.L. (2000). A β toxicity in Alzheimer's Disease. In: Chesselet, M. F. (Eds.), *Molecular mechanisms of neurodegenerative diseases*. Totowa, NJ: Humana Press, Inc., pp. 1–49.
- Klein, W.L. (2002). Abeta toxicity in Alzheimer's disease: globular oligomers (ADDLs) as new vaccine and drug targets. *Neurochem. Int.* 41:345–352.

- Klein, W.L., Stine, W.B., Jr., and Teplow, D.B. (2004). Small assemblies of unmodified amyloid beta-protein are the proximate neurotoxin in Alzheimer's disease. *Neurobiol. Aging* 25:569–580.
- Klunk, W.E., Debnath, M.L., and Pettegrew, J.W. (1994). Development of small molecule probes for the beta-amyloid protein of Alzheimer's disease. *Neurobiol. Aging* 15:691–698.
- Klunk, W.E., Engler, H., Nordberg, A., Bacskaï, B.J., Wang, Y., Price, J.C., Bergstrom, M., Hyman, B.T., Langstrom, B., and Mathis, C.A. (2003). Imaging the pathology of Alzheimer's disease: amyloid-imaging with positron emission tomography. *Neuroimaging Clin. N. Am.* 13:781–789.
- Kotilinek, L.A., Bacskaï, B., Westerman, M., Kawarabayashi, T., Younkin, L., Hyman, B.T., Younkin, S., and Ashe, K.H. (2002). Reversible memory loss in a mouse transgenic model of Alzheimer's disease. *J. Neurosci.* 22:6331–6335.
- Kuo, Y.M., Emmerling, M.R., Vigo-Pelfrey, C., Kasunic, T.C., Kirkpatrick, J.B., Murdoch, G.H., Ball, M.J., and Roher, A.E. (1996). Water-soluble Abeta (N-40, N-42) oligomers in normal and Alzheimer disease brains. *J. Biol. Chem.* 271:4077–4081.
- Lacor, P.N., Viola, K.L., Fernandez, S., Velasco, P.T., Bigio, E.H., and Klein, W.L. (2003). Targeting of synapses and memory-linked IEG protein Arc by ADDLs. *Soc. Neurosci. Abstr.* 29:945.14.
- Lacor, P.N., Buniel, M.C., Cain, P.C., Chang, L., Lambert, M.P., and Klein, W.L. (2004). Synaptic targeting by Alzheimer's related Abeta oligomers. OASIS—Online Abstract Submission and Invitation System™ P3–319.
- Lambert, M.P., Stevens, G., Sabo, S., Barber, K., Wang, G., Wade, W., Krafft, G., Holzman, T.F., and Klein, W.L. (1994). Beta/A4-evoked degeneration of differentiated SH-SY5Y human neuroblastoma cells. *J. Neurosci. Res.* 39: 377–385.
- Lambert, M.P., Barlow, A.K., Chromy, B.A., Edwards, C., Freed, R., Liosatos, M., Morgan, T.E., Rozovsky, I., Trommer, B., Viola, K.L., Wals, P., Zhang, C., Finch, C.E., Krafft, G.A., and Klein, W.L. (1998). Diffusible, nonfibrillar ligands derived from Abeta1–42 are potent central nervous system neurotoxins. *Proc. Natl. Acad. Sci. USA* 95: 6448–6453.
- Lambert, M.P., Viola, K.L., Chromy, B.A., Chang, L., Morgan, T.E., Yu, J., Venton, D.L., Krafft, G.A., Finch, C.E., and Klein, W.L. (2001). Vaccination with soluble Abeta oligomers generates toxicity-neutralizing antibodies. *J. Neurochem.* 79:595–605.
- Lashuel, H.A., Hartley, D.M., Petre, B.M., Wall, J.S., Simon, M.N., Walz, T., and Lansbury, P.T., Jr. (2003). Mixtures of wild-type and a pathogenic (E22G) form of Abeta40 *in vitro* accumulate protofibrils, including amyloid pores. *J. Mol. Biol.* 332:795–808.
- Lindersson, E., Beedholm, R., Hojrup, P., Moos, T., Gai, W., Hendil, K.B., and Jensen, P.H. (2004). Proteasomal inhibition by alpha-synuclein filaments and oligomers. *J. Biol. Chem.* 279:12924–12934.
- Lo Bianco, C., Ridet, J.L., Schneider, B.L., Deglon, N., and Aebischer, P. (2002). alpha-Synucleinopathy and selective dopaminergic neuron loss in a rat lentiviral-based model of Parkinson's disease. *Proc. Natl. Acad. Sci. USA* 99:10813–10818.
- Longo, V.D., Viola, K.L., Klein, W.L., and Finch, C.E. (2000). Reversible inactivation of superoxide-sensitive aconitase in Abeta1–42-treated neuronal cell lines. *J. Neurochem.* 75:1977–1985.
- Lorenzo, A., and Yankner, B.A. (1994). Beta-amyloid neurotoxicity requires fibril formation and is inhibited by congo red. *Proc. Natl. Acad. Sci. USA* 91:12243–12247.
- Lyford, G.L., Yamagata, K., Kaufmann, W.E., Barnes, C.A., Sanders, L.K., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Lanahan, A.A., and Worley, P.F. (1995). Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. *Neuron* 14:433–445.
- Malenka, R.C. (2003). The long-term potential of LTP. *Nat. Rev. Neurosci.* 4:923–926.
- Masliah, E., Rockenstein, E., Veinbergs, I., Mallory, M., Hashimoto, M., Takeda, A., Sagara, Y., Sisk, A., and Mucke, L. (2000). Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. *Science* 287:1265–1269.
- Mattson, M. P. (2004). Metal-catalyzed disruption of membrane protein and lipid signaling in the pathogenesis of neurodegenerative disorders. *Ann. N. Y. Acad. Sci.* 1012:37–50.
- Mesulam, M.M. (1999). Neuroplasticity failure in Alzheimer's disease: bridging the gap between plaques and tangles. *Neuron* 24:521–529.
- Morgan, D., Diamond, D.M., Gottschall, P.E., Ugen, K.E., Dickey, C., Hardy, J., Duff, K., Jantzen, P., DiCarlo, G., Wilcock, D., Connor, K., Hatcher, J., Hope, C., Gordon, M., and Arendash, G.W. (2000). ABeta peptide vaccination prevents memory loss in animal model of Alzheimer's disease. *Nature* 408:982–985.
- Mucke, L., Masliah, E., Yu, G.Q., Mallory, M., Rockenstein, E.M., Tatsuno, G., Hu, K., Kholodenko, D., Johnson-Wood, K., and McConlogue, L. (2000). High-level neuronal expression of abeta 1–42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J. Neurosci.* 20:4050–4058.

- Nam, J.M., Thaxton, C.S., and Mirkin, C.A. (2003). Nanoparticle-based bio-bar codes for the ultrasensitive detection of proteins. *Science* 301:1884–1886.
- Narayanan, S., Bosl, B., Walter, S., and Reif, B. (2003). Importance of low-oligomeric-weight species for prion propagation in the yeast prion system Sup35/Hsp104. *Proc. Natl. Acad. Sci. USA* 100:9286–9291.
- Norris, E.H., Giasson, B.I., and Lee, V.M. (2004). Alpha-synuclein: normal function and role in neurodegenerative diseases. *Curr. Top. Dev. Biol.* 60:17–54.
- Oda, T., Pasinetti, G.M., Osterburg, H.H., Anderson, C., Johnson, S.A., and Finch, C.E. (1994). Purification and characterization of brain clusterin. *Biochem. Biophys. Res. Commun.* 204:1131–1136.
- Oda, T., Wals, P., Osterburg, H.H., Johnson, S.A., Pasinetti, G.M., Morgan, T.E., Rozovsky, I., Stine, W.B., Snyder, S.W., and Holzman, T.F. (1995). Clusterin (apoJ) alters the aggregation of amyloid beta-peptide (A beta 1–42) and forms slowly sedimenting A beta complexes that cause oxidative stress. *Exp. Neurol.* 136:22–31.
- Pike, C.J., Walencewicz, A.J., Glabe, C.G., and Cotman, C.W. (1991). *In vitro* aging of beta-amyloid protein causes peptide aggregation and neurotoxicity. *Brain Res.* 563:311–314.
- Pike, C.J., Burdick, D., Walencewicz, A.J., Glabe, A.J., and Cotman, C.W. (1993). Neurodegeneration induced by beta-amyloid peptides *in vitro*: the role of peptide assembly state. *J. Neurosci.* 13:1676–1687.
- Pitschke, M., Prior, R., Haupt, M., and Riesner, D. (1998). Detection of single amyloid beta-protein aggregates in the cerebrospinal fluid of Alzheimer's patients by fluorescence correlation spectroscopy [see comments]. *Nat. Med.* 4:832–834.
- Podlisny, M.B., Ostaszewski, B.L., Squazzo, S.L., Koo, E.H., Rydell, R.E., Teplow, D.B., and Selkoe, D.J. (1995). Aggregation of secreted amyloid beta-protein into sodium dodecyl sulfate-stable oligomers in cell culture. *J. Biol. Chem.* 270:9564–9570.
- Price, D.L., Tanzi, R.E., Borchelt, D.R., and Sisodia, S.S. (1998). Alzheimer's disease: genetic studies and transgenic models. *Annu. Rev. Genet.* 32:461–493.
- Reixach, N., Deechongkit, S., Jiang, X., Kelly, J.W., and Buxbaum, J.N. (2004). Tissue damage in the amyloidoses: transthyretin monomers and nonnative oligomers are the major cytotoxic species in tissue culture. *Proc. Natl. Acad. Sci. USA* 101:2817–2822.
- Rhee, S.K., Quist, A.P., and Lal, R. (1998). Amyloid beta protein-(1–42) forms calcium-permeable, Zn²⁺-sensitive channel. *J. Biol. Chem.* 273:13379–13382.
- Rial Verde, E.M., Worley, P., Malinow, R., and Cline, H.T. (2003). The immediate-early gene Arc regulates AMPA receptor trafficking. *Soc. Neurosci. Abstr.* 29:55.7.
- Savage, M.J., Lin, Y.G., Ciallella, J.R., Flood, D.G., and Scott, R.W. (2002). Activation of c-Jun N-terminal kinase and p38 in an Alzheimer's disease model is associated with amyloid deposition. *J. Neurosci.* 22:3376–3385.
- Scheibel, A.B., and Tomiyasu, U. (1978). Dendritic sprouting in Alzheimer's presenile dementia. *Exp. Neurol.* 60:1–8.
- Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Liao, Z., Lieberburg, I., Motter, R., Mutter, L., Soriano, F., Shopp, G., Vasquez, N., Vandever, C., Walker, S., Wogulis, M., Yednock, T., Games, D., and Seubert, P. (1999). Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Lett. Nat.* 400:173–177.
- Selkoe, D.J. (2002). Alzheimer's disease is a synaptic failure. *Science* 298:789–791.
- Souillac, P.O., Uversky, V.N., and Fink, A.L. (2003). Structural transformations of oligomeric intermediates in the fibrillation of the immunoglobulin light chain LEN. *Biochemistry* 42:8094–8104.
- Stine, W.B., Jr., Dahlgren, K.N., Krafft, G.A., and LaDu, M.J. (2003). *In vitro* characterization of conditions for amyloid-beta peptide oligomerization and fibrillogenesis. *J. Biol. Chem.* 278:11612–11622.
- Terry, R.D. (1994). Neuropathological changes in Alzheimer disease. *Prog. Brain Res.* 101:383–390.
- Terry, R.D. (1999). In: Terry, R.D., Katzman, R., Bick, K.L., and Sisodia, S.S. (Eds.), *Alzheimer disease*, 2nd ed. Philadelphia: Lippincott, Williams, and Wilkins.
- Tezuka, T., Umemori, H., Akiyama, T., Nakanishi, S., and Yamamoto, T. (1999). PSD-95 promotes Fyn-mediated tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit NR2A. *Proc. Natl. Acad. Sci. USA* 96:435–440.
- Tong, L., Thornton, P.L., Balazs, R., and Cotman, C.W. (2001). Beta-amyloid-(1–42) impairs activity-dependent cAMP-response element-binding protein signaling in neurons at concentrations in which cell survival is not compromised. *J. Biol. Chem.* 276:17301–17306.
- Volles, M.J., and Lansbury, P.T., Jr. (2002). Vesicle permeabilization by protofibrillar alpha-synuclein is sensitive to Parkinson's disease-linked mutations and occurs by a pore-like mechanism. *Biochemistry* 41:4595–4602.
- Walsh, D.M., Lomakin, A., Benedek, G.B., Condron, M.M., and Teplow, D.B. (1997). Amyloid beta-protein fibrillogenesis. Detection of a protofibrillar intermediate. *J. Biol. Chem.* 272:22364–22372.

- Walsh, D.M., Hartley, D.M., Kusumoto, Y., Fezoui, Y., Condron, M.M., Lomakin, A., Benedek, G.B., Selkoe, D.J., and Teplow, D.B. (1999). Amyloid beta-protein fibrillogenesis. Structure and biological activity of protofibrillar intermediates. *J. Biol. Chem.* 274:25945–25952.
- Walsh, D.M., Klyubin, I., Fadeeva, J.V., Cullen, W.K., Anwyl, R., Wolfe, M.S., Rowan, M.J., and Selkoe, D.J. (2002). Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* 416:535–539.
- Wang, H.W., Pasternak, J.F., Kuo, H., Ristic, H., Lambert, M.P., Chromy, B., Viola, K.L., Klein, W.L., Stine, W.B., Krafft, G.A., and Trommer, B.L. (2002). Soluble oligomers of beta amyloid (1–42) inhibit long-term potentiation but not long-term depression in rat dentate gyrus. *Brain Res.* 924:133–140.
- Wang, Q., Walsh, D.M., Rowan, M.J., Selkoe, D.J., and Anwyl, R. (2004a). Block of long-term potentiation by naturally secreted and synthetic amyloid beta-peptide in hippocampal slices is mediated via activation of the kinases c-Jun N-terminal kinase, cyclin-dependent kinase 5, and p38 mitogen-activated protein kinase as well as metabotropic glutamate receptor type 5. *J. Neurosci.* 24:3370–3378.
- Wang, Z., Chang, L., Klein, W.L., Thatcher, G.R., and Venton, D.L. (2004b). Per-6-substituted-per-6-deoxy beta-cyclodextrins inhibit the formation of beta-amyloid peptide derived soluble oligomers. *J. Med. Chem.* 47:3329–3333.
- Xu, J., Kao, S.Y., Lee, F.J., Song, W., Jin, L.W., and Yankner, B.A. (2002). Dopamine-dependent neurotoxicity of alpha-synuclein: a mechanism for selective neurodegeneration in Parkinson disease. *Nat. Med.* 8:600–606.
- Yankner, B.A. (1996). Mechanisms of neuronal degeneration in Alzheimer's disease [review]. *Neuron* 16:921–932.
- Yao, Z., Drieu, K., and Papadopoulos, V. (2001). The Ginkgo biloba extract EGb 761 rescues the PC12 neuronal cells from beta-amyloid-induced cell death by inhibiting the formation of beta-amyloid-derived diffusible neurotoxic ligands. *Brain Res.* 889:181–190.
- Ying, S.W., Futter, M., Rosenblum, K., Webber, M.J., Hunt, S.P., Bliss, T.V., and Bramham, C.R. (2002). Brain-derived neurotrophic factor induces long-term potentiation in intact adult hippocampus: requirement for ERK activation coupled to CREB and upregulation of Arc synthesis. *J. Neurosci.* 22:1532–1540.
- Yu, J., Bakhos, L., Chang, L., Holterman, M.J., Klein, W.L., and Venton, D.L. (2002). Per-6-substituted beta-cyclodextrin libraries inhibit formation of beta-amyloid-peptide (A beta)-derived, soluble oligomers. *J. Mol. Neurosci.* 19: 51–55.
- Zhu, M., Han, S., Zhou, F., Carter, S.A., and Fink, A.L. (2004). Annular oligomeric amyloid intermediates observed by in situ atomic force microscopy. *J. Biol. Chem.* 279:24452–24459.
- Zhukareva, V., Trojanowski, J.Q., and Lee, V.M. (2004). Assessment of pathological tau proteins in frontotemporal dementias: qualitative and quantitative approaches. *Am. J. Geriatr. Psychiatry* 12:136–145.

Glycosaminoglycans, Proteoglycans, and Conformational Disorders

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1. Abstract

Increasing evidence exists to support a potentially crucial role for proteoglycans, in particular heparan sulfate proteoglycans, in the pathophysiology of many protein conformational disorder diseases. This chapter will focus on emerging evidence that supports a role for proteoglycans in the regulation of protein conformation in amyloid diseases, with the result being that proteoglycans are attractive therapeutic targets for these diseases. Amyloid diseases in both the nervous system and nonneural tissues share a common property with proteoglycans, especially heparan sulfate proteoglycans, being associated with amyloid fibrils coincident with the time amyloid fibrils are formed in the disease process. The binding of heparan sulfate proteoglycans to the specific amyloid-associated protein has been shown in many cases to lead to conformational changes in the amyloid-associated protein, with an introduction of β -sheet structure to the amyloid protein. Heparan sulfate proteoglycans have also been demonstrated to accelerate the formation of amyloid oligomers, protofibrils, and fibrils, as well as impart resistance to proteolytic degradation of the amyloid fibrils. Heparan sulfate proteoglycans therefore appear to play a critical role in the progression of amyloid diseases, and an ability to prevent heparan sulfate binding to amyloid-associated proteins may allow both an inhibition of the formation of new amyloid, as well as promote the clearance of existing amyloid.

2. Biochemical Properties of Proteoglycans and Glycosaminoglycans

Before embarking on a discussion of the role of proteoglycans in protein misfolding and aggregation in conformational disorders and amyloid diseases, it is useful to review the biochemical properties of proteoglycans and their associated glycosaminoglycan (GAG) chains. Proteoglycans are families of proteins that are defined based on the covalent attachment of GAG chains to the protein core (Yanagishita and Hascall, 1992; David, 1993). GAG chains are comprised of long, linear, unbranched repeating disaccharide units that undergo extensive modifications that regulate their biological activity (Hook *et al.*, 1984; Gallagher *et al.*, 1986). The most important modification to GAG chains is sulfation of specific monosaccharides in the GAG chain. The enzymes that catalyze these modifications are located in the Golgi apparatus. There are three types of GAG chain that are covalently attached to a polypeptide chain to produce a proteoglycan, with the nomenclature for proteoglycans being based on the type of GAG chain attached to the protein core.

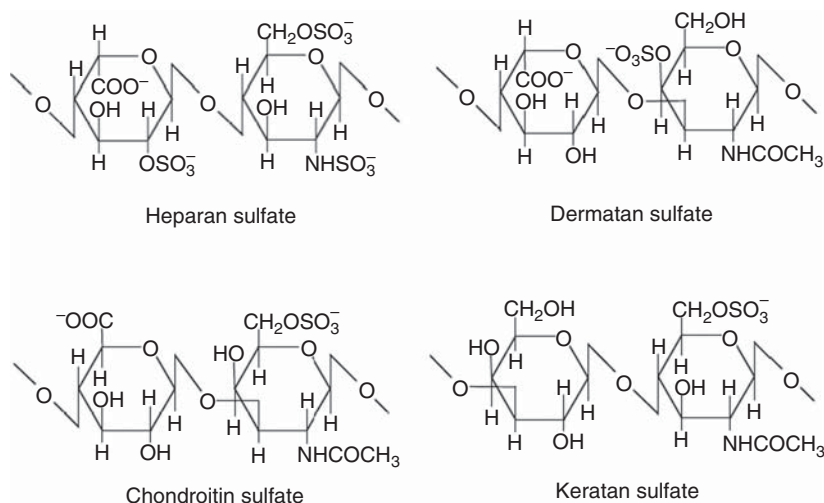


Figure 5-1. The structure of HS-GAG, CS-GAG, DS-GAG, and KS-GAG is illustrated. The first sugar group in the disaccharide is uronic acid (either glucuronic or iduronic acid), except in the case of KS-GAG, which lacks uronic acid. The second sugar group in the disaccharide is an amino sugar, either N-acetylglucosamine in the case of HS-GAG and KS-GAG, or N-acetylgalactosamine in the case of CS-GAG and DS-GAG. Potential sulfation sites are also indicated, although it should be noted that the sulfation of GAG residues is variable and highly regulated, contributing to the specificity of GAG binding to proteins.

Thus, heparan sulfate proteoglycans (HSPGs) contain heparan sulfate GAG chains (HS-GAGs), chondroitin sulfate proteoglycans (CSPGs) contain either chondroitin sulfate or dermatan sulfate GAG chains (CS-GAGs), and keratan sulfate proteoglycans (KSPGs) contain keratan sulfate GAG chains (KS-GAGs). Hybrid proteoglycans have also been identified, which can contain different classes of GAG chains attached to the same protein core (i.e., HS- and CS-GAGs). For the purpose of this chapter we will focus our discussion primarily on HSPGs, as it appears that it is this class of proteoglycan that contributes most to the regulation of protein misfolding in protein conformational disorders.

The structure of disaccharide subunits that comprise the major GAG chains is shown in Figure 5-1. Important differences in the structure of the different GAGs, which contribute to their specific functions, include differences in the amino sugars represented in CS-GAGs and HS-GAGs (N-acetylgalactosamine versus N-acetylglucosamine, respectively), and differences in the pattern of sulfation in the individual GAG chains. CS-GAGs typically contain higher levels of sulfate residues than HS-GAGs, with one sulfate per repeating disaccharide. HS-GAGs are more variable in their sulfation, when compared to CS-GAGs, with both the uronic acid group and amino sugar group being sulfated in HS-GAGs (Figure 5-1). However, HS-GAGs contain regions of low sulfation (N-acetylated domains) that are spaced between regions of higher sulfation. The variable, higher sulfated domains are involved in the binding of HS-GAGs to protein ligands, providing specific HSPGs the ability to mediate distinct functions and bind distinct classes of heparin-binding proteins (Aviezer *et al.*, 1994; Sanderson *et al.*, 1994; Cotman *et al.*, 1999; Herndon *et al.*, 1999; Knox *et al.*, 2002). In addition, changes in the pattern of sulfation of HS-GAGs from the same HSPG protein core, isolated from different tissues, has been shown to result in different binding activities for the HSPGs (Kato

et al., 1994; Sanderson *et al.*, 1994). Furthermore, a change in the sulfation pattern of HS-GAGs from the same HSPG protein core during development has been shown to modulate the binding activity of specific HSPGs (Nurcombe *et al.*, 1993; Brickman *et al.*, 1998).

As shown in **Figure 5-2; see color insert**, there are currently five known classes of HSPGs in vertebrates. The syndecans and glypicans exist as multiple gene products with distinct cellular and tissue distribution, and are primarily membrane-associated proteoglycans (Bernfield *et al.*, 1992; Lander *et al.*, 1996; De Cat and David, 2001). However, syndecans can be cleaved by extracellular proteases to release a functional ectodomain (Bernfield *et al.*, 1992) and glypicans are attached to the plasma membrane via a glycosylphosphatidylinositol anchor, thus allowing release of glypicans from the plasma membrane. Perlecan was the first identified extracellular matrix/basement membrane HSPG (Murdoch *et al.*, 1992), and collagen XVIII is the newest member of the basement membrane HSPGs (Halfter *et al.*, 1998; Saarela *et al.*, 1998). Agrin is an HSPG that was initially identified as a basement membrane HSPG (Tsen *et al.*, 1995), but recent studies have shown that agrin also exists via alternative splicing as a transmembrane HSPG (Burgess *et al.*, 2000; Neumann *et al.*, 2001). Agrin shares with some syndecans the ability to be expressed as a hybrid proteoglycan, containing more than one type of GAG chain. Thus, syndecans and agrin can contain both HS-GAG and CS-GAG chains attached to their protein core (Rapraeger *et al.*, 1985; Winzen *et al.*, 2003).

3. Neurodegenerative Diseases Are Protein Conformational Disorders

Recent studies have begun to shed light on similarities between the various and quite distinctive neurodegenerative diseases, with these diseases all being protein conformational disorders (Soto, 2001, 2003). These diseases include Alzheimer’s disease (AD), Parkinson’s disease (PD), the polyglutamine expansion diseases that include Huntington’s disease, the prion diseases that include the transmissible spongiform encephalopathies (TSEs), and amyotrophic lateral sclerosis. The properties of these seemingly diverse neurodegenerative diseases, that are thought to share a common pathological mechanism, are shown in Table 5-1. Interestingly, these diseases involve distinct cellular proteins that affect specific classes of neurons in the central nervous system, with the resulting abnormal

Table 5-1. Properties of neurodegenerative, protein conformational disorder diseases

Disease	Protein	Affected brain region	Location of protein aggregates
Alzheimer’s	β-amyloid, tau	Cerebral cortex, hippocampus	Extracellular, cytoplasmic microvasculature
Parkinson’s; dementia with Lewy bodies	α-synuclein	Substantia nigra	Cytoplasmic
Prion diseases; TSE; Creutzfeldt-Jakob	Prion protein (PrP ^C and PrP ^{Sc})	Variable	Extracellular
Huntington’s; polyglutamine expansion diseases	Huntingtin	Cerebral cortex, Striatum	Nuclear
Amyotrophic lateral Sclerosis	Superoxide dismutase	Brainstem, cerebral cortex, spinal cord	Cytoplasmic

protein aggregates also exhibiting quite distinct cellular locations. The common thread to these neurodegenerative diseases is that their respective proteins undergo misfolding leading to protein aggregation and amyloid formation, and ultimately cell death (Soto, 2003). However, another shared property of these diseases may include a potential role for proteoglycans in the underlying pathophysiology. It has long been recognized that proteoglycans, especially HSPGs, are associated with all of the pathological lesions of AD (Snow *et al.*, 1987a, 1989a, 1990a, 1996; Young *et al.*, 1989; Su *et al.*, 1992; DeWitt *et al.*, 1993). There exists convincing support for an association of HSPGs with the lesions found in prion diseases (Snow *et al.*, 1989b, 1990b; McBride *et al.*, 1998), and recent studies have suggested that HSPGs may contribute to the abnormal protein aggregation of α -synuclein in PD (Cohlberg *et al.*, 2002). Likewise, it has been suggested that proteoglycans, in this case CSPGs, may be associated with reactive astrocytes and lesions in Huntington's disease (DeWitt *et al.*, 1994). Thus, it is tempting to speculate that the neurodegenerative conformational protein disorders may not only share a common mechanism of protein misfolding leading to disease progression, but that, in addition, proteoglycans may contribute to the abnormal protein folding associated with these diverse neurodegenerative diseases.

3.1. AD

The best characterized example of a neurodegenerative disease that is a protein conformational disorder disease, with strong evidence for proteoglycans playing a key role in the pathophysiology of the disease, is AD. The disease is characterized by a progressive loss of memory and other cognitive functions, with the majority of cases being sporadic in nature with an onset in later decades of life (Selkoe, 1991). Confirmation of AD in a patient is usually made post mortem, based on the histopathological characteristics of the disease, which include senile plaques, cerebral amyloid angiopathy (microvascular plaques), and neurofibrillary tangles. The primary protein constituent of senile and microvascular plaques is β -amyloid peptide ($A\beta$), a 40–42-amino acid peptide that is a hallmark of AD and that is produced by proteolytic processing of the amyloid precursor polypeptide (Glenner and Wong, 1984; Masters *et al.*, 1985; Tanzi *et al.*, 1987). The primary protein component of neurofibrillary tangles is hyperphosphorylated tau, a microtubule-associated protein that with hyperphosphorylation in AD forms intracellular paired helical filaments (Grundke-Iqbal *et al.*, 1986; Kosik *et al.*, 1986; Wood *et al.*, 1986). Numerous other proteins have been shown to be associated with these three types of lesions in AD, and importantly, HSPGs are a component of all three lesion types (Snow *et al.*, 1987a; Goedert *et al.*, 1996; Spillantini *et al.*, 1999; Verbeek *et al.*, 1999; Cotman *et al.*, 2000).

The earliest documentation of the presence of proteoglycans in senile plaques in AD was made by the Snow and Kisilevsky laboratories, which showed that HS-GAGs were associated with $A\beta$ plaques (Snow *et al.*, 1987a). These studies initially employed cationic dyes to demonstrate the presence of sulfated GAGs in AD lesions (Snow *et al.*, 1987a, 1989a; Young *et al.*, 1989) and subsequently used antisera to HS-GAGs to confirm the presence of these molecules in AD (Snow *et al.*, 1990a). As additional reagents to proteoglycans became available it was shown that all classes of proteoglycans, HSPG, CSPG, and KSPG, were present in $A\beta$ plaques (Snow *et al.*, 1992, 1996; DeWitt *et al.*, 1993). However, it appears that HSPGs are likely the most abundant proteoglycan component of $A\beta$ plaques and other lesions in AD. HSPGs are associated with early, immature plaques that are called diffuse plaques (Snow *et al.*, 1990a; Su *et al.*, 1992; Cotman *et al.*, 2000), as well as mature dense-core plaques (Su *et al.*, 1992; Donahue *et al.*, 1999; Verbeek *et al.*, 1999; Cotman *et al.*, 2000). Conversely, CSPGs and KSPGs have been shown to associate later during the course of the disease with mature plaques, and are associated with the periphery of plaques (Snow *et al.*, 1992, 1996).

Recent studies have focused on identifying the relevant HSPG(s) that is associated with AD lesions, using specific antisera to the known classes of HSPG. Using antisera to the various classes of HSPG it has been reported that all classes of HSPG are associated with A β senile plaques and microvasculature plaques (Snow *et al.*, 1990a; Donahue *et al.*, 1999; Verbeek *et al.*, 1999; Cotman *et al.*, 2000). However, recent studies by Verbeek *et al.* (1999) have called into question the localization of perlecan to AD lesions, with the possibility being that early studies on the association of perlecan with AD lesions may have used antisera with crossreactivity to other HSPGs. A careful comparison of the distribution of perlecan, agrin, collagen XVIII, glypican-1, and syndecans 1–3 in AD has shown a variable distribution for the different HSPG molecules in AD lesions (Verbeek *et al.*, 1999; Van Horssen *et al.*, 2003). Agrin appears to be the most widespread HSPG in AD lesions, being associated with senile plaques, microvasculature plaques, and neurofibrillary tangles (Verbeek *et al.*, 1999). Perlecan was only observed in normal blood vessels (Verbeek *et al.*, 1999), and collagen XVIII is primarily associated with classic senile plaques and A β plaques in the microvasculature (Van Horssen *et al.*, 2002). Analysis of cell surface HSPGs has shown that the syndecans and glypican-1 are more variable in their distribution in AD lesions, with glypican-1 being present in classic senile plaques and microvasculature plaques, but not tangles (Van Horssen *et al.*, 2001, 2003). The syndecans are not associated with A β lesions in the microvasculature, are for the most part absent from tangles, and exhibit variability in expression in senile plaques (Van Horssen *et al.*, 2001, 2003).

Based on analyses demonstrating that HSPGs, or HS-GAGs, are associated with AD lesions even at early pathological stages of the disease, it was predicted that HSPGs might contribute to the generation of AD lesions. As part of this hypothesis, it was also predicted that HSPGs would bind A β and regulate the formation of amyloid fibrils by A β . Studies by Snow *et al.* (1994) have shown that perlecan can bind A β and promote the fibrillation of A β both *in vitro* and *in vivo*. In view of the recent work of Verbeek *et al.* (1999) suggesting that perlecan may not be a physiologically relevant HSPG in AD, our laboratory has investigated the ability of agrin to bind A β and regulate A β fibrillation. We have shown that agrin binds fibrillar A β with high affinity, and based on ThioT fluorescence analysis and electron microscopy analysis agrin promotes and accelerates A β fibrillation (Cotman *et al.*, 2000).

In light of the association of agrin with diffuse plaques, and an ability to promote A β fibrillation, we predicted that agrin might function as a chaperone protein that could bind A β and modulate A β folding. A current model for protein misfolding in protein conformational disorder diseases is that as a result of either mutations in the polypeptide sequence, or the binding of pathological chaperones, the folding state of the protein can be perturbed such that a misfolded protein is generated (Soto, 2001, 2003) (**Figure 5-3; see color insert**). The misfolded protein subsequently undergoes aggregation leading to amyloid formation, with a resulting gain of toxic activity or loss of normal biological function (**Figure 5-3; see color insert**). This ultimately leads to the cascade of events that in neurodegenerative diseases results in neuron cell death. The ability of HSPGs, in particular HS-GAGs, to affect A β peptide conformation has been explored. McLaurin *et al.* (1999a) used a marine sponge HSPG to show that HS-GAGs induce a conformational change in A β peptide, changing A β from a peptide with random structure to primarily a β -sheet structure. These investigators also examined the activities of different GAG chains on altering A β conformation, and found that although HS-GAGs alter A β conformation, CS-GAGs are also capable of inducing β -sheet conformation in A β (McLaurin *et al.*, 1999b). To identify the central nervous system HSPG that might mediate changes in A β structure during the pathophysiology of AD, we tested whether agrin acts as a chaperone that regulates A β conformation. We analyzed A β structure in the absence or presence of agrin using circular dichroism, with these experiments demonstrating that although A β is a random structured peptide, agrin induces a change in A β structure to a peptide containing primarily a β -sheet

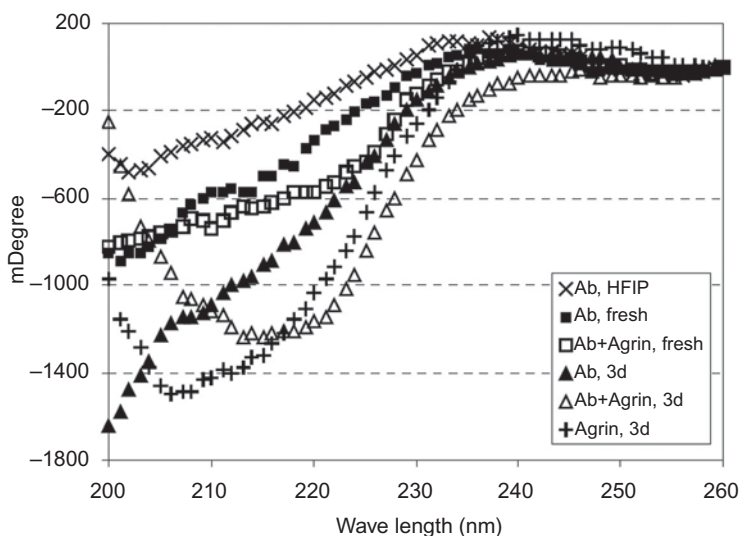


Figure 5-4. Analysis of the effect of agrin on the A β structure using circular dichroism. A β in the absence or presence of agrin was analyzed by circular dichroism, with either freshly incubated A β and agrin, or A β incubated with agrin for 3 days. It can be seen that although A β remains a random structured peptide, agrin appears to have an immediate effect on the A β structure, and by 3 days of incubation has converted the A β from a random-structured peptide to a peptide rich in β -sheet structure.

structure (Figure 5-4). These data are consistent with our collective experimental data, with agrin possessing the capability to bind A β , induce the introduction of a β -sheet structure in A β , with the subsequent acceleration of A β fibrillation and the formation of amyloids. It is also noteworthy that previous studies have shown that both HS-GAG and CS-GAG chains can modulate A β folding and protein structure (McLaurin *et al.*, 1999b), and because agrin can potentially contain both classes of GAG chains, agrin may be able to differentially influence functions such as protein folding via diverse types of GAG chains attached to its protein core.

If HSPGs are involved in the induction of misfolding in amyloidogenic proteins such as A β , this raises the question of whether specific HS-GAG structures might be involved in the regulation of protein misfolding. As described below, we have observed that only a subset of highly glycosylated agrin binds to α -synuclein, raising the interesting possibility that a specific HS-GAG structure might possess amyloidogenic activity by being able to bind to amyloid proteins. In AD, there is evidence for changes in HS-GAG sulfation in neurons in response to ApoE4 (Bonay and Avila, 2001), with ApoE4 being a known susceptibility gene for AD (Roses, 1996). An analysis of HS-GAG structure in AD brain revealed no differences in HS-GAG structure between age-matched control and AD brain (Lindahl *et al.*, 1995), although an analysis of skin fibroblasts from AD patients revealed changes in HS-GAG sulfation (Zebrower *et al.*, 1992), suggesting alterations in HS sulfotransferases in AD (Zebrower and Kieras, 1993). It is important to consider these studies in terms of the HSPGs being analyzed, as total HS-GAGs from brain or fibroblasts were analyzed, without isolation of specific HSPGs. It remains possible that subsets of HSPGs, such as agrin, are preferentially affected in AD, with only subsets of HS-GAG chains also undergoing the modification in structure. Thus, it will

be important to analyze the structure of specific HSPGs in amyloid tissues to ascertain if the structure of HS-GAG chains on these HSPGs is altered in the disease state.

Interestingly, when considering strategies to overcome the accumulation of A β in the AD brain, targets for therapy have included preventing A β production and formation of amyloids, as well as clearance of A β from the AD brain. Early studies from Snow's laboratory showed that HSPG, in this case perlecan, prevented proteolytic degradation of A β fibrils (Snow and Wight, 1989). These studies were confirmed by Gupta-Bansal *et al.* (1995), who extended these analyses to show that cartilage CSPG could inhibit proteolytic degradation of A β . Our laboratory has also shown that agrin impairs protease-mediated degradation of A β fibrils, using both nonspecific proteases (Cotman *et al.*, 2000) as well as proteases such as plasmin that are thought to play an important role in A β clearance from brain (I.-H. Liu, L. Mitchell, and G. J. Cole, unpublished data). Therefore, an ability to target HSPG association with AD lesions, or to prevent HSPG binding to the A β peptide, may be beneficial to the disease process by affecting A β biology at several different biochemical levels. Recent studies have begun to explore the use of heparan sulfate mimetics as therapies for AD, using both *in vitro* and *in vivo* paradigms (Gervais *et al.*, 2001; Zhu *et al.*, 2001; Kisilevsky and Szarek, 2002; Kisilevsky *et al.*, 2003). These compounds are exhibiting promise, as they appear to have the potential to affect A β fibrillogenesis, neurotoxicity, and clearance (Bergamaschini *et al.*, 2004).

3.2. PD

Parkinson's disease is the second most common neurodegenerative disease after AD, and recent studies have begun to provide insight into how PD and AD may share many properties, despite affecting different brain regions and gene products. PD is a neurodegenerative disease characterized by loss of dopaminergic neurons in the substantia nigra. To date, at least three genes are implicated in the etiology of PD: α -synuclein (PARK1), parkin (PARK2), and UCH-L1. Interestingly, a peptide fragment of α -synuclein, termed a non-A β component of amyloid plaques, has been shown to copurify with amyloid plaques from AD (Ueda *et al.*, 1993). Aggregates of α -synuclein have been associated with numerous neurodegenerative diseases, termed synucleinopathies (Duda *et al.*, 2000), that include PD, and α -synuclein has been shown to be a component of lesions in the amygdala and entorhinal cortex of familial AD patients (Lippa *et al.*, 2001). The aggregation and fibrillation of α -synuclein, a 140-amino acid synaptic protein, is thought to be a causative factor for PD (Polymeropoulos *et al.*, 1997). α -Synuclein is a major molecular component of Lewy bodies and Lewy neurites (Spillantini *et al.*, 1997), the primary pathological lesions in PD, with fibrillar α -synuclein being found in these lesions. The aggregation/fibrillation of α -synuclein is potentiated by a number of different mechanisms, including mutations in the α -synuclein gene (Narhi *et al.*, 1999). α -Synuclein fibrillation is also potentiated by interactions with sulfated macromolecules, with the extent and rate of fibrillation being increased by heparin (Cohlberg *et al.*, 2002). The presence of heparitinase-sensitive FGF binding sites in Lewy bodies suggests the presence of HSPGs in Lewy bodies (Perry *et al.*, 1992), raising the possibility that HSPGs also contribute to the etiology and pathogenesis of PD. The fact that AD and PD both share the property of protein aggregation/fibrillation potentially contributing to the etiology of the disease is of interest, especially with regard to the role of HSPGs in the potential aggregation of A β and α -synuclein.

Based on this information our laboratory, in collaboration with Tony Fink's laboratory, initiated studies to determine whether agrin is capable of binding to α -synuclein, and whether agrin regulates α -synuclein fibrillation. In these studies we have shown that agrin binds to α -synuclein and decreases the solubility of α -synuclein in solution (Liu *et al.*, 2005). Interestingly, we have shown that a subset of agrin that is more highly glycosylated binds to α -synuclein. These data suggest that longer HS-GAG chains or more sulfated HS-GAG chains on agrin may be involved in binding to α -synuclein.

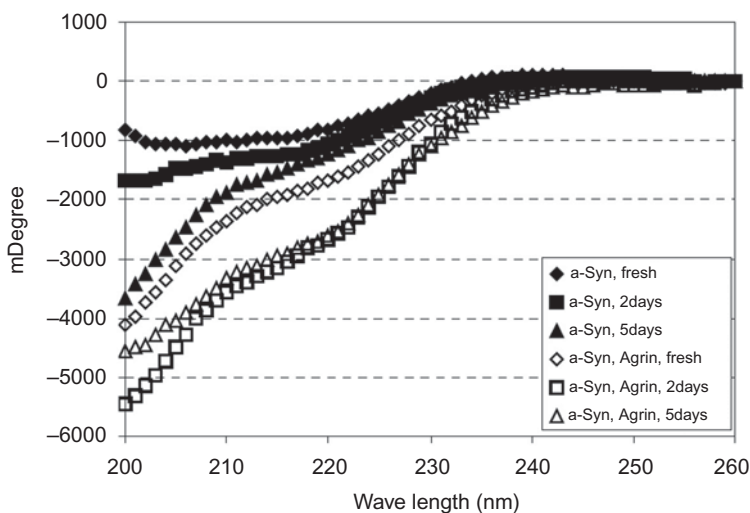


Figure 5-5. Analysis of the effect on agrin on α -synuclein structure using circular dichroism. α -Synuclein incubated with or without agrin for up to 5 days was analyzed by circular dichroism. These data show that α -synuclein incubated without agrin for 5 days resembles in structure fresh α -synuclein coincubated with agrin. A further change in α -synuclein structure occurs with α -synuclein coincubated with agrin for 2 or 5 days. The shift in spectra suggests that α -synuclein rapidly acquires a β -sheet structure in the presence of agrin.

Our studies have also shown by circular dichroism that agrin induces a conformational change in α -synuclein, which is normally an unstructured protein, with the β -sheet being introduced in α -synuclein by agrin (Figure 5-5). Agrin's ability to modulate α -synuclein fibrillation was also assessed by electron microscopy and Thio T fluorescence, and these data indicate that agrin significantly accelerates α -synuclein fibrillation as measured by Thio T fluorescence, decreasing the lag time (nucleation) for fibril formation, and decreasing the half-life for fibrillation (Liu *et al.*, 2005). These data provide additional support for the importance of HSPGs in protein misfolding associated with protein conformational disorders, and now demonstrates that PD protein aggregation and amyloid formation may be regulated by HSPGs such as agrin.

One caveat that must be considered with studies examining the role of HSPGs in PD is that lesions and protein aggregates are located intracellularly in affected neurons. However, it is worth noting that heparinase-sensitive FGF-binding sites have been identified in Lewy bodies, the intracellular neuronal lesion of PD (Perry *et al.*, 1992). CSPGs have also been localized to Lewy bodies in PD (DeWitt *et al.*, 1994), although what, if any, role they may play in the pathology of PD is unknown. In addition, there is a wealth of evidence that demonstrates an intracellular localization of HSPGs, especially in disease states (Perry *et al.*, 1992; Su *et al.*, 1992; Odawara *et al.*, 1998; Donahue *et al.*, 1999; Roskams *et al.*, 1999; Verbeek *et al.*, 1999; Cotman *et al.*, 2000; Lundquist and Schmidtchen, 2001). As already mentioned, there is evidence for the intracellular localization of HSPGs in neurodegenerative diseases such as AD (Su *et al.*, 1992; Donahue *et al.*, 1999; Verbeek *et al.*, 1999; Cotman *et al.*, 2000) and PD (Perry *et al.*, 1992), and it has also been shown that HSPGs associate with Pick bodies in Pick disease (Odawara *et al.*, 1998). It has also been shown recently that HSPGs colocalize

intracellularly in neurons with BACE1 (Scholefield *et al.*, 2003). It is therefore conceivable that in pathological conditions associated with a disease such as PD, agrin and/or other HSPGs become localized intracellularly, where they can function as a pathological chaperone to affect α -synuclein aggregation.

3.3. Prion Diseases

A third class of neurodegenerative diseases classified as protein conformational disorders are the prion diseases. These diseases are categorized as TSEs, based on their histopathological properties of spongiform degeneration of brain tissue (Prusiner, 1991; Budka *et al.*, 1995; Hetz and Soto, 2003; Koster *et al.*, 2003). These diseases are also characterized by extensive astrogliosis, microglial activation, and extensive degeneration of neurons (Budka *et al.*, 1995; Williams *et al.*, 1997). Primary symptoms of the disease include a progressive dementia, ataxia, and loss of fine control of body homeostasis. The prion diseases are comprised of a family of related diseases that can be familial, sporadic, or infectious in nature. The familial forms of the disease include Creutzfeldt-Jakob disease (CJD), Gertmann-Straussler-Sheinker syndrome (GSS), and fatal familial insomnia (Prusiner and Scott, 1997). The sporadic forms of the disease include some forms of CJD. The infectious forms of prion diseases include scrapie and bovine spongiform encephalopathy (BSE), kuru, and new variant Creutzfeldt-Jakob disease (vCJD), which is thought to occur as a result of human exposure to BSE (Collinge, 1999, 2001; Bruce, 2000; Hetz and Soto, 2003). All of the prion diseases involve the prion protein (PrP), which has a normal cellular component (PrP^c), which is a membrane-associated protein that is widely expressed in neural and nonneural tissues (Borchelt *et al.*, 1990; Prusiner, 1998). In the disease state PrP^c is converted to a misfolded variant referred to as PrP^{sc}, whereby a conformational change in PrP^c results in a decrease in the α -helical content of PrP^c and a pronounced increase in β -sheet content in the misfolded PrP^{sc} protein (Caughey *et al.*, 1991; Pan *et al.*, 1993; Thompson and Barrow, 2002; Hetz and Soto, 2003).

The mechanism(s) that contributes to misfolding of PrP^c is currently open to debate, but the association of proteoglycans with PrP and prion lesions has raised the question of whether proteoglycans might also serve a chaperone role in the pathophysiology of prion diseases. Early experiments to assess the association of proteoglycans or GAGs with prion lesions employed basic stains, such as Alcian blue, to demonstrate that sulfated GAGs were present in amyloid plaques from CJD, GSS, and kuru brains, as well as in the plaques of hamsters with experimental scrapie (Snow *et al.*, 1989b). An extension of these studies demonstrated that perlecan core protein and HS-GAGs could be localized to CJD, GSS, and scrapie amyloid plaques (Snow *et al.*, 1990b).

The association of HSPGs with immature prion amyloid plaques in scrapie mice (McBride *et al.*, 1998) suggests that HSPGs may contribute to the fibrillation of PrP^c; much as has been postulated for HSPGs in other neurodegenerative protein conformational disorders. Support for this hypothesis has been obtained by experiments employing analogs of sulfated glycans, and PrP^{sc} infected neuroblastoma cells. Congo red is a cationic dye that is used in many amyloid diseases to identify amyloid deposits, with this dye being a sulfonated molecule that resembles the sulfated glycans that are found in proteoglycans (Caughey and Raymond, 1993). Congo red inhibits the accumulation of PrP^{sc} in scrapie-infected neuroblastoma cells (Caughey and Race, 1992; Caughey and Raymond, 1993), suggesting that the binding of sulfated glycans to PrP^c could contribute to the misfolding of this protein. PrP^c has been shown to bind heparin-like molecules (Gabizon *et al.*, 1993; Caughey *et al.*, 1994), and sulfated polyanions such as pentosan sulfate have been shown to inhibit the accumulation of PrP^{sc} in scrapie-infected neuroblastoma cells (Gabizon *et al.*, 1993; Caughey and Race, 1994; Caughey *et al.*, 1994). The effect of these sulfated glycans on PrP^{sc} accumulation appears to be the result of the sulfated glycans preventing PrP^c conversion to PrP^{sc}, and not due to these molecules downregulating

cellular levels of PrP^c (Caughey and Raymond, 1993; Gabizon *et al.*, 1993). This has led to the hypothesis that cellular HSPGs, via binding to PrP^c, may regulate the misfolding of PrP^c such that the normal cellular protein becomes misfolded, leading to the formation of protease-resistant PrP^{sc} that forms insoluble protein aggregates and amyloid plaques. Recent studies employing heparan sulfate mimetics to assess the role of HS-GAGs in the conversion of PrP^c to PrP^{sc} in prion-infected neuroblastoma cells have shown a pronounced reduction in PrP^{sc} protein levels in these cells, with an activity approximately fivefold greater than pentosan sulfate (Schonberger *et al.*, 2003). These heparan mimetics eliminate PrP^{sc} from the prion-infected neuroblastoma cells, with their effects being due to blocking PrP^c to PrP^{sc} conversion and not as a result of decreasing PrP^c synthesis or attachment of PrP^c to lipid rafts. These studies therefore raise the interesting possibility that a cellular HSPG acts as a receptor for PrP^c, with this HSPG playing a direct role in the conversion of normal cellular PrP^c to the PrP^{sc} protein variant. Consistent with this hypothesis, treatment of prion-infected neuroblastoma cells with β -D-xyloside, an inhibitor of HS-GAG biosynthesis, results in a pronounced reduction in PrP^{sc} levels in the prion-infected cells (Ben-Zaken *et al.*, 2003). Treatment of these cells with heparanase III, an enzyme that degrades a subset of HS-GAG chains, also resulted in a reduction in PrP^{sc} (Ben-Zaken *et al.*, 2003). These results provide possibly the best evidence to date that HS-GAG chains play a crucial role in the conversion of PrP^c to PrP^{sc}. It will be of interest to determine which HSPG in the brain is capable of modulating the conversion of PrP^c to PrP^{sc}, and whether HSPGs are able to induce the conformational changes in PrP^c that lead to the introduction of β -sheet structure and protein misfolding.

4. Proteoglycans Contribute to Protein Misfolding in Conformational Protein Disorders Outside the Nervous System

Experimental evidence for a contribution of proteoglycans to the pathophysiology of amyloid diseases was first obtained with amyloid diseases that were systemic, and not neural, in origin. Early studies on the polysaccharide composition of amyloid tissues showed that amyloid-laden spleen and liver were enriched in heparin-like molecules (Hass, 1942; Bitter and Muir, 1966). Subsequent studies demonstrated that a variety of amyloid tissues contained sulfated GAGs, including inflammation-associated amyloid (AA amyloid), immunoglobulin light chain amyloid (AL), islet amyloid of type 2 diabetes (IAPP), amyloid of medullary carcinoma, inherited cutaneous amyloid, and senile cardiac amyloid (Snow *et al.*, 1987b; Young *et al.*, 1989, 1992; Magnus *et al.*, 1991). The association of GAGs with these various amyloids appeared to coincide with the formation of amyloid, lending the first evidence that sulfated glycans may have a direct role in amyloidosis. In the following sections we will describe the recent progress that has been made in the characterization of the specific HSPGs that are involved in some systemic amyloidoses, and will provide insight into possible mechanisms by which HSPGs contribute to amyloidosis in these diseases.

4.1. Type 2 Diabetes

Type 2 diabetes is a disease with increasing incidence due to the increased prevalence of obesity in society. A pathologic hallmark of the majority of individuals with type 2 diabetes is the presence of islet amyloid (Kahn *et al.*, 1999). Amyloid deposits in a type 2 diabetics pancreas lead to a loss of β -cell mass (Kahn *et al.*, 1999), with a concomitant loss of insulin production by the pancreas. The amyloid protein that comprises islet amyloid is the β -cell protein amylin, referred to as islet

amyloid polypeptide (IAPP) in type 2 diabetes (Kahn *et al.*, 1999). Amylin is a normal β -cell secretory product that is cosecreted with insulin from pancreatic β -cells (Kahn *et al.*, 1990; Hartter *et al.*, 1991). Amylin is synthesized as a pro-peptide in β -cells, with its N-terminus cleaved from the pro-peptide prior to secretion with insulin (Nishi *et al.*, 1989). It has been demonstrated that in type 2 diabetes β -cells pro-insulin is not processed normally to insulin (Kahn and Halban, 1997), and accordingly, recent studies have also shown that IAPP is not processed normally in type 2 diabetes β -cells (Park and Verchere, 2001).

Islet amyloid polypeptide is not the only protein that is associated with amyloid deposits in type 2 diabetes. Both ApoE and HSPGs have been shown to be associated with islet amyloid (Young *et al.*, 1992; Kahn *et al.*, 1999), indicating that islet amyloid resembles other amyloid fibrils from other amyloid diseases. The source of HSPGs in islet amyloid is likely to be pancreatic β -cells, as these cells have been shown to synthesize and secrete extracellular matrix HSPGs (Potter-Perigo *et al.*, 2003). Although the identity of the HSPG secreted by β -cells was not determined, based on its molecular mass and other properties the HSPG is likely to be either perlecan or agrin. Immunohistochemical experiments using antisera to perlecan have demonstrated immunoreactivity for perlecan in islet amyloid (Young *et al.*, 1992), and currently it is unknown whether agrin is expressed in the pancreas. Perlecan has been shown to enhance IAPP amyloid formation, and perlecan has been shown to bind amylin via its HS-GAG chains (Castillo *et al.*, 1998). In addition, the ability of GAG chains to augment IAPP fibril formation has been shown to exhibit the highest activity for HS-GAGs, when compared to CS-GAGs (Castillo *et al.*, 1998).

Interestingly, the observed disruption in normal processing of IAPP in type 2 diabetes is likely to play a crucial role in the ability of HSPGs to modulate the formation of IAPP amyloid fibrils. The abnormal processing of proamylin in pancreatic β -cells in type 2 diabetes results in the retention of the pro-peptide N-terminus on IAPP, which has been shown to contain a heparin-binding site that is rendered inactive with normal processing of proamylin (Park and Verchere, 2001). The IAPP that is localized to islet amyloid deposits has been shown to retain this N-terminal region (Park and Verchere, 2001), providing a basis for the binding of IAPP to HSPG. Although perlecan has been shown to augment amyloid fibril formation by IAPP, as measured by enhanced ThioT fluorescence, which is suggestive of increased β -sheet structure in IAPP (Castillo *et al.*, 1998), studies have not been conducted to demonstrate conclusively that HSPGs induce a conformational change in IAPP, leading to misfolding of the protein and introduction of β -sheet structure. Despite this caveat, it appears that islet amyloid represents another example of a protein conformational disorder whereby HSPGs may contribute a crucial regulatory role to the acquisition of the misfolded protein conformation.

4.2. Inflammation-Associated AA Amyloidosis

Inflammation-associated amyloidosis is characterized by deposition of amyloid deposits in various organs (i.e., liver and spleen), occurring in response to chronic inflammation. The amyloid deposits are comprised of the amyloidogenic serum amyloid A (AA) protein, which is derived from a novel high-density lipoprotein apolipoprotein called serum amyloid A protein (SAA; Ansin and Kisilevsky, 1999). SAA proteins, which exist as three isoforms, exhibit a dramatic increase in concentration during the first 24 hours of the inflammation process (McAdam and Sipe, 1976). Of the SAA isoforms, SAA₂ is the most likely SAA protein to undergo aggregation, and is the precursor of the amyloidogenic AA protein (McCubbin *et al.*, 1988). SAA₂ has been shown to bind HS-GAGs (McCubbin *et al.*, 1988; Ansin and Kisilevsky, 1999), but not CS-GAGs (Ansin and Kisilevsky, 1999). Importantly, HS-GAG binding to SAA₂, but not SAA₁, has been shown by circular dichroism to induce a conformational change in the SAA₂ protein, with a change from an α -helix to a β -sheet structure (McCubbin *et al.*, 1988). Because HS-GAGs and HSPGs have been shown to colocalize

with AA amyloid deposits (Snow *et al.*, 1987b), these studies suggest that HSPGs may have an integral role in AA amyloidogenesis. Providing support for this suggestion, it has been shown that there is increased deposition of HS-GAGs in AA liver and spleen, as well as a change in HS-GAG structure in AA spleen and liver (Lindahl and Lindahl, 1997). In addition, in experimental AA a rapid increase in HS-GAG and HSPG biosynthesis has been demonstrated, with these increases being detectable even before amyloid deposits are visible (Stenstad *et al.*, 1994). Likewise, perlecan has been shown to be associated with AA amyloid deposits (Snow *et al.*, 1991), and perlecan mRNA levels are significantly upregulated in experimental AA, again prior to the detection of amyloid deposits (Ailles *et al.*, 1993). Collectively, these data provide support for the hypothesis that the association of HSPGs with the amyloidogenic AA protein can modulate amyloidosis in AA, and that HSPGs are capable of inducing a conformational change in SAA₂, leading to protein misfolding. Thus, inflammation-associated amyloidosis can also be classified as a protein conformational disorder, with HSPG binding to the amyloid protein possibly playing a direct role in the misfolding of the protein that leads to amyloidogenesis.

4.3. β_2 -Microglobulin-Related Amyloidosis

β_2 -Microglobulin-related amyloidosis is a hemodialysis-associated disease that is a complication of long-term kidney dialysis (Koch, 1992). β_2 -Microglobulin is the major structural protein component of the amyloid fibrils in this disease, although other proteins that include ApoE, HSPGs, and CSPGs are associated with the β_2 -microglobulin amyloid fibrils (Ohishi *et al.*, 1990; Ohashi *et al.*, 1995). In the disease state β_2 -microglobulin undergoes partial denaturation, with a change in protein folding, with this conformation of β_2 -microglobulin favoring the formation of protein aggregates and amyloid fibrils (Chiti *et al.*, 2001). Because the earliest deposition of β_2 -microglobulin amyloid occurs in cartilage tissue, which is particularly proteoglycan-rich, this has led to the suggestion that proteoglycans may also play a key contributory role to amyloidosis in this disease. β_2 -Microglobulin has been shown to bind heparin with high affinity (Ohashi *et al.*, 2002), and heparin binding has been shown to enhance β_2 -microglobulin amyloid fibril formation and ThioT fluorescence, which is suggestive of augmented β -sheet structure (Yamaguchi *et al.*, 2003). The binding of intact proteoglycans to β_2 -microglobulin, which included CSPGs (biglycan and decorin) and KSPGs, also produced increased ThioT fluorescence and amyloid fibril formation (Yamaguchi *et al.*, 2003). These latter data are of interest, because β_2 -microglobulin amyloid deposits occur in tissues (i.e., cartilage) that are rich in CSPG and KSPG, therefore indicating that these classes of proteoglycans may also play a crucial role in modulating the conformation of amyloid proteins in protein conformational disorders.

5. Conclusions

The association of sulfated polysaccharides with amyloid lesions has been recognized for greater than 5 decades, and only in the last 15 years have studies begun to shed light on the types of sulfated polysaccharides that are localized to amyloid deposits in various diseases. Our current understanding that virtually every amyloid disease studied to date contains proteoglycans (Ancsin, 2003) has raised our awareness of how these complex macromolecules may play a crucial role in the pathophysiology of amyloid diseases and protein conformational disorders. Recent studies have implicated, in most cases, that HSPGs as the likely class of proteoglycan that may regulate amyloidosis. Emerging evidence provides strong support for the ability of HSPGs to bind to a variety of amyloid proteins and to mediate the alteration in protein conformation that leads to a misfolded, β -

sheet-rich protein that either acquires a toxic activity or loses its normal biological function. The targeting of HS-GAG chains and HSPG function has also emerged as an attractive therapeutic strategy. Importantly, the ability to target HS-GAG interactions with amyloid proteins may allow a limited number of therapeutic strategies to target a large number of seemingly unrelated protein conformation disorder and amyloid diseases.

6. Abbreviations

AA	Inflammation-associated amyloid
A β	β -Amyloid peptide
AD	Alzheimer's disease
AL	Immunoglobulin light chain amyloid
BSE	Bovine spongiform encephalopathy
CJD	Creutzfeldt-Jakob disease
CS-GAG	Chondroitin sulfate GAG
CSPG	Chondroitin sulfate proteoglycan
DS-GAG	Dermatan sulfate GAG
GAG	Glycosaminoglycan
GSS	Gertmann-Straussler-Sheinker syndrome
HSPG	Heparan sulfate proteoglycans
HS-GAG	Heparan sulfate GAG
IAPP	Islet amyloid polypeptide in type 2 diabetes
KS-GAG	Keratan sulfate GAG
KSPG	Keratan sulfate proteoglycans
PD	Parkinson's disease
PrP	Prion protein
SAA	Serum amyloid A protein
Thio T	Thioflavine T
TSE	Transmissible spongiform encephalopathies

References

- Ailles, L., Kisilevsky, R., and Young, I.D. (1993). Induction of perlecan gene expression preceded amyloid formation during experimental murine AA amyloidogenesis. *Lab. Invest.* 69:443–448.
- Ancsin, J.B. (2003). Amyloidogenesis: historical and modern observations point to heparan sulfate proteoglycans as a major culprit. *Amyloid* 10:67–79.
- Ancsin, J.B., and Kisilevsky, R. (1999). The heparin/heparan sulfate-binding site on Apo-serum Amyloid A. Implications for the therapeutic intervention of amyloidosis. *J. Biol. Chem.* 274:7172–7181.
- Aviezer, D., Levy, E., Safran, M., Svahn, C., Buddecke, E., Schmidt, A., David, G., Vlodavsky, I., and Yayon, A. (1994). Differential structural requirements of heparin and heparan sulfate proteoglycans that promote binding of basic fibroblast growth factor to its receptor. *J. Biol. Chem.* 269:114–121.
- Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M.T., Spring, J., Gallo, R.L., and Lose, E.J. (1992). Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans. *Annu. Rev. Cell Biol.* 8:365–393.
- Ben-Zaken, O., Tzaban, S., Tal, Y., Horonchik, L., Esko, J.D., Vlodavsky, I., and Taraboulos, A. (2003). Cellular heparan sulfate participates in the metabolism of prions. *J. Biol. Chem.* 278:40041–40049.

- Bergamaschini, L., Rossi, E., Storini, C., Pizzimnetti, S., Distaso, M., Perego, C., De Luigi, A., Vergani, C., and De Simoni, M.G. (2004). Peripheral treatment with enoxaparin, a low molecular weight heparin, reduces plaques and β -amyloid accumulation in a mouse model of Alzheimer's disease. *J. Neurosci.* 24:4181–4186.
- Bitter, T., and Muir, H. (1966). Mucopolysaccharides of whole human spleens in generalized amyloidosis. *J. Clin. Invest.* 45:963–975.
- Bonay, P., and Avila, J. (2001). Apolipoprotein E4 stimulates sulfation of glycosaminoglycans in neural cells. *Biochim. Biophys. Acta* 1535:217–220.
- Borchelt, D.R., Scott, M., Taraboulos, A., Stahl, N., and Prusiner, S.B. (1990). Scrapie and cellular prion proteins differ in the kinetics of synthesis and topology in cultured cells. *J. Cell Biol.* 110:743–752.
- Brickman, Y.G., Ford, M.D., Gallagher, J.T., Nurcombe, V., Bartlett, P.F., and Turnbull, J.E. (1998). Structural modification of fibroblast growth factor-binding heparan sulfate at a determinative stage of neural development. *J. Biol. Chem.* 273:4350–4359.
- Bruce, M.E. (2000). "New variant" Creutzfeldt-Jakob disease and bovine spongiform encephalopathy. *Nat. Med.* 6:258–259.
- Budka, H., Aguzzi, A., Brown, P., Brucher, J.M., Bugiani, O., Gullotta, F., Haltia, M., Hauw, J.J., Ironside, J.W., Jellinger, K., Kretschmar, H.A., Lantos, P.L., Masullo, C., Schlote, W., Tateishi, J., and Weller, R.O. (1995). Neuropathological diagnostic criteria for Creutzfeldt-Jakob disease (CJD) and other human spongiform encephalopathies (prion diseases). *Brain Pathol.* 5:459–466.
- Burgess, R.W., Skarnes, W.C., and Sanes, J.R. (2000). Agrin isoforms with distinct amino termini: differential expression, localization, and function. *J. Cell Biol.* 151:41–52.
- Castillo, G.M., Cummings, J.A., Yang, W., Judge, M.E., Sheardown, M.J., Rimvall, K., Hansen, J.B., and Snow, A.D. (1998). Sulfate content and specific glycosaminoglycan backbone of perlecan are critical for perlecan's enhancement of islet amyloid peptide (amylin) fibril formation. *Diabetes* 47:612–620.
- Caughey, B., and Race, R.E. (1992). Potent inhibition of scrapie-associated PrP accumulation by Congo red. *J. Neurochem.* 59:768–771.
- Caughey, B., and Race, R.E. (1994). Scrapie-associated PrP accumulation and its inhibition: revisiting the amyloid-glycosaminoglycan connection. *Ann. N. Y. Acad. Sci.* 724:290–295.
- Caughey, B., and Raymond, G.J. (1993). Sulfated polyanion inhibition of scrapie-associated PrP accumulation in cultured cells. *J. Virol.* 67:643–650.
- Caughey, B.W., Dong, A., Bhat, K.S., Ernst, D., Hayes, S.F., and Caughey, W.S. (1991). Secondary structure analysis of the scrapie-associated protein PrP 27–30 in water by infrared spectroscopy. *Biochemistry* 30:7672–7680.
- Caughey, B., Brown, K., Raymond, G.J., Katzenstein, G.E., and Thresher, W. (1994). Binding of the protease-sensitive form of prion protein PrP to sulfated glycosaminoglycan and Congo Red. *J. Virol.* 68:2135–2141.
- Chiti, F., Mangione, P., Andreola, A., Giorgetti, S., Stefani, M., Dobson, C.M., Bellotti, V., and Taddei, N. (2001). Detection of two partially structured species in the folding process of the amyloidogenic protein beta-2-microglobulin. *J. Mol. Biol.* 307:379–391.
- Cohlberg, J.A., Li, J., Uversky, V.N., and Fink, A.L. (2002). Heparin and other glycosaminoglycans stimulate the formation of amyloid fibrils from α -synuclein in vitro. *Biochemistry* 41:1502–1511.
- Collinge, J. (1999). Variant Creutzfeldt-Jakob disease. *Lancet* 354:317–323.
- Collinge, B. (2001). Prion diseases of humans and animals: their causes and molecular basis. *Annu. Rev. Neurosci.* 24:519–550.
- Cotman, S.L., Halfter, W., and Cole, G.J. (1999). Identification of extracellular matrix ligands for the heparan sulfate proteoglycan agrin. *Exp. Cell Res.* 249:54–64.
- Cotman, S.L., Halfter, W., and Cole, G.J. (2000). Agrin binds to β -amyloid (A β), accelerates A β fibril formation, and is localized to A β deposits in Alzheimer's Disease brain. *Mol. Cell. Neurosci.* 15:183–198.
- David, G. (1993). Integral membrane heparan sulfate proteoglycans. *FASEB J.* 7:1023–1030.
- DeCat, B., and David, G. (2001). Developmental roles of the glypicans. *Semin. Cell Dev. Biol.* 12:117–125.
- DeWitt, D.A., Silver, J., Canning, D.R., and Perry, G. (1993). Chondroitin sulfate proteoglycans are associated with the lesions of Alzheimer's disease. *Exp. Neurol.* 121:149–152.
- DeWitt, D.A., Richey, P.L., Praprotnik, D., Silver, J., and Perry, G. (1994). Chondroitin sulfate proteoglycans are a common component of neuronal inclusions and astrocytic reaction in neurodegenerative diseases. *Brain Res.* 656: 205–209.
- Donahue, J.E., Berzin, T.M., Rafii, M.S., Glass, D.J., Yancopoulos, G.D., Fallon, J.R., and Stopa, E.G. (1999). Agrin in Alzheimer's disease: altered solubility and abnormal distribution within microvasculature and brain parenchyma. *Proc. Natl. Acad. Sci. USA* 96:6468–6472.
- Duda, J.E., Lee, V. M.-Y., and Trojanowski, J.Q. (2000). Neuropathology of synuclein aggregates: new insights into mechanisms of neurodegenerative diseases. *J. Neurosci. Res.* 61:121–127.

- Gabizon, R., Meiner, Z., Halimi, M., and Ben-Sasson, S.A. (1993). Heparin-like molecules bind differentially to prion-proteins and change their intracellular metabolic fate. *J. Cell Physiol.* 157:319–325.
- Gallagher, J.T., Lyon, M., and Steward, W.P. (1986). Structure and function of heparan sulfate proteoglycans. *Biochem. J.* 236:313–325.
- Gervais, F., Chalifour, R., Garceau, D., Kong, X., Laurin, J., McLaughlin, R., Morissette, C., and Paquette, J. (2001). Glycosaminoglycan mimetics: a therapeutic approach to cerebral angiopathy. *Amyloid* 8:28–35.
- Glenner, G.G., and Wong, C.W. (1984). Alzheimer's Disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* 120:885–890.
- Goedert, M., Jakes, R., Spillantini, M.G., Hasegawa, M., Smith, M.J., and Crowther, R.A. (1996). Assembly of microtubule-associated protein tau into Alzheimer-like filaments induced by sulphated glycosaminoglycans. *Nature* 383:550–553.
- Grunde-Iqbal, I., Iqbal, K., Quinlan, M., Tung, Y.C., Zaidi, M.S., and Wisniewski, H.M. (1986). Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. *J. Biol. Chem.* 261:6084–6089.
- Gupta-Bansal, R., Frederickson, R.C.A., and Brunden, K.R. (1995). Proteoglycan-mediated inhibition of A β proteolysis. A potential cause of senile plaque accumulation. *J. Biol. Chem.* 270:18666–18671.
- Halfter, W., Dong, S., Schurer, B., and Cole, G.J. (1998). Collagen XVIII is a basement membrane heparan sulfate proteoglycan. *J. Biol. Chem.* 273:25404–25412.
- Hartter, E., Svoboda, T., Ludvik, B., Schuller, M., Lell, B., Kuenburg, E., Brunnbauer, M., Woloszczuk, W., and Prager, R. (1991). Basal and stimulated plasma levels of pancreatic amylin indicate its co-secretion with insulin in humans. *Diabetologia* 34:52–54.
- Hass, G. (1942). Studies of amyloid II: the isolation of a polysaccharide from amyloid-bearing tissues. *Arch. Pathol.* 34:92–105.
- Herndon, M.E., Stipp, C.S., and Lander, A.D. (1999). Interactions of neural glycosaminoglycans and proteoglycans with protein ligands: assessment of selectivity, heterogeneity and the participation of core proteins in binding. *Glycobiology* 2:143–155.
- Hetz, C., and Soto, C. (2003). Protein misfolding and disease: the case of prion disorders. *Cell. Mol. Life Sci.* 60:133–143.
- Hook, M., Kjellen, L., Johansson, S., and Robinson, J. (1984). Cell-surface glycosaminoglycans. *Annu. Rev. Biochem.* 53:847–869.
- Iozzo, R.V. (2001). Heparan sulfate proteoglycans: intricate molecules with intriguing functions. *J. Clin. Invest.* 108:165–167.
- Kahn, S.E., and Halban, P.A. (1997). Release of incompletely processed proinsulin is the cause of the disproportionate proinsulinemia of NIDDM. *Diabetes* 46:1725–1732.
- Kahn, S.E., D'Alessio, D.A., Schwartz, M.W., Fujimoto, W.Y., Ensink, J.W., Taborsky, G.J., Jr., and Porte, D., Jr. (1990). Evidence of cosecretion of islet amyloid polypeptide and insulin by β -cells. *Diabetes* 39:634–638.
- Kahn, S.E., Andrikopoulos, S., and Verchere, C.B. (1999). Islet amyloid. A long-recognized but underappreciated pathological feature of type 2 diabetes. *Diabetes* 48:241–253.
- Kato, M., Wang, H., Bernfield, M., Gallagher, J.T., and Turnbull, J.E. (1994). Cell surface syndecan-1 on distinct cell types differs in fine structure and ligand binding of its heparan sulfate chains. *J. Biol. Chem.* 269:18881–18890.
- Kisilevsky, R., and Szarek, W.A. (2002). Novel glycosaminoglycan precursors as anti-amyloid agents, Part II. *J. Mol. Neurosci.* 19:45–50.
- Kisilevsky, R., Szarek, W.A., Ancsin, J., Bhat, S., Li, Z., and Marone, S. (2003). Novel glycosaminoglycan precursors as anti-amyloid agents, Part III. *J. Mol. Neurosci.* 20:291–297.
- Knox, S., Merry, C., Stringer, S., Melrose, J., and Whitelock, J. (2002). Not All perlecan are created equal. *J. Biol. Chem.* 277:14657–14665.
- Koch, K.M. (1990). Dialysis-related amyloidosis. *Kidney Int.* 41:1416–1429.
- Kosik, K.S., Joachim, C.L., and Selkoe, D.J. (1986). Microtubule-associated protein tau is a major antigenic component of paired helical filaments in Alzheimer disease. *Proc. Natl. Acad. Sci. USA* 83:4044–4048.
- Koster, T., Singh, K., Zimmermann, M., and Gruys, E. (2003). Emerging therapeutic agents for transmissible spongiform encephalopathies: a review. *J. Vet. Pharm. Ther.* 26:315–326.
- Lander, A.D., Stipp, C.S., and Ivins, J.K. (1996). The glypican family of heparan sulfate proteoglycans: major cell-surface proteoglycans of the developing nervous system. *Perspect. Dev. Neurobiol.* 3:347–358.
- Lindahl, B., and Lindahl, U. (1997). Amyloid-specific heparan sulfate from human liver and spleen. *J. Biol. Chem.* 272:26091–26094.
- Lindahl, B., Eriksson, L., and Lindahl, U. (1995). Structure of heparan sulphate from human brain, with special regard to Alzheimer's disease. *Biochem. J.* 306:177–184.

- Lippa, C.F., Schmidt, M.L., Lee, V.M.-Y., and Trojanowski, J.Q. (2001). α -Synuclein in familial Alzheimer disease. *Arch. Neurol.* 58:1817–1820.
- Liu, I.-H., Uversky, V.N., Munishkina, L.A., Fink, A.L., Halfter, W., and Cole, G.J. (2005). Agrin binds α -synuclein and modulates α -synuclein fibrillation. *Glycobiology*, 15:1320–1331.
- Lundquist, K., and Schmidtchen, A. (2001). Immunohistochemical studies on proteoglycan expression in normal skin and chronic ulcers. *Br. J. Dermatol.* 144:254–259.
- Magnus, J.H., Stenstad, T., Kolset, S.O., and Husby, G. (1991). Glycosaminoglycans in extracts of cardiac amyloid fibrils from familial amyloid cardiomyopathy of Danish origin related to variant transthyretin Met 111. *Scand. J. Immunol.* 34:63–69.
- Masters, C.L., Simms, G., Weinman, N.A., Multhaup, G., McDonald, B.L., and Beyreuther, K. (1985). Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc. Natl. Acad. Sci. USA* 82:4245–4249.
- McAdam, K.P., and Sipe, J.D. (1976). Murine model for human secondary amyloidosis: genetic variability of the acute-phase serum protein SAA response to endotoxins and casein. *J. Exp. Med.* 144:1121–1127.
- McBride, P.A., Wilson, M.I., Eikelenboom, P., Tunstall, A., and Bruce, E.M. (1998). Heparan sulfate proteoglycan is associated with amyloid plaques and neuroanatomically targeted PrP pathology throughout the incubation period of scrapie-infected mice. *Exp. Neurol.* 149:447–454.
- McCubbin, W.D., Kay, C.M., Narindrasorasak, S., and Kisilevsky, R. (1988). Circular-dichroism studies on two murine serum amyloid A proteins. *Biochem. J.* 256:775–783.
- McLaurin, J., Franklin, T., Kuhns, W.J., and Fraser, P.E. (1999a). A sulfated proteoglycan aggregation factor mediates amyloid- β -peptide fibril formation and neurotoxicity. *Amyloid* 6:233–243.
- McLaurin, J., Franklin, T., Zhang, X., Deng, J., and Fraser, P.E. (1999b). Interactions of Alzheimer amyloid- β peptides with glycosaminoglycans. Effects on fibril nucleation and growth. *Eur. J. Biochem.* 266:1101–1110.
- Murdoch, A.D., Dodge, G.R., Cohen, I., Tuan, R.S., and Iozzo, R.V. (1992). Primary structure of the heparan sulfate proteoglycan from basement membrane (HSPG2/perlecan): a chimeric molecule with multiple domains homologous to the low density lipoprotein receptor, laminin, neural cell adhesion molecules, and epidermal growth factor. *J. Biol. Chem.* 267:8544–8557.
- Narhi, L., Wood, S.J., Steavenson, S., Jiang, Y., Wu, G.M., Anafi, D., Kaufman, S.A., Martin, F., Sitney, K., Denis, P., Louis, J.C., Wypych, J., Biere, A.L., and Citron, M. (1999). Both familial Parkinson's disease mutations accelerate alpha-synuclein aggregation. *J. Biol. Chem.* 274:9843–9846.
- Neumann, F.R., Bittcher, G., Annies, M., Schumacher, B., Kroger, S., and Ruegg, M.A. (2001). An alternative amino-terminus expressed in the central nervous system converts agrin to a type II transmembrane protein. *Mol. Cell. Neurosci.* 17:208–225.
- Nishi, M., Sanke, T., Seino, S., Eddy, R.L., Fan, Y.-S., Byers, M.G., Shows, T.B., Bell, G.I., and Steiner, D.F. (1989). Human islet amyloid polypeptide gene: complete nucleotide sequence, chromosomal localization and evolutionary history. *Mol. Endocrinol.* 3:1775–1781.
- Nurcombe, V., Ford, M.D., Wildschut, J.A., and Bartlett, P.F. (1993). Developmental regulation of neural response to FGF-1 and FGF-2 by heparan sulfate proteoglycan. *Science* 260:103–106.
- Odawara, T., Iseki, E., Li, F., Kosaka, K., and Ikeda, K. (1998). Heparan sulfate proteoglycans recognize ghost Pick bodies. *Neurosci. Lett.* 242:120–122.
- Ohashi, K., Hara, M., Yanagishita, M., Kawai, R., Tachibana, S., and Ogura, Y. (1995). Proteoglycans in hemodialysis-related amyloidosis. *Virchows Arch.* 427:49–59.
- Ohashi, K., Kisilevsky, R., and Yanagishita, M. (2002). Affinity binding of glycosaminoglycans with beta(2)-microglobulin. *Nephron* 90:158–168.
- Ohishi, H., Skinner, M., Sato-Araki, N., Okuyama, T., Gejyo, F., Kimura, A., Cohen, A.S., and Schmid, K. (1990). Glycosaminoglycans of the hemodialysis-associated carpal synovial amyloid and of amyloid-rich tissues and fibrils of heart, liver, and spleen. *Clin. Chem.* 36:88–91.
- Pan, K.M., Baldwin, M., Njuyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R.J., Cohen, F.E., and Prusiner, S.B. (1993). Conversion of alpha-helices into β -sheets features in the formation of scrapie prion proteins. *Proc. Natl. Acad. Sci. USA* 90:10962–10966.
- Park, K., and Verchere, C.B. (2001). Identification of a heparin binding domain in the N-terminal cleavage site of pro-islet amyloid polypeptide. Implications for islet amyloid formation. *J. Biol. Chem.* 276:16611–16616.
- Perry, G., Richey, P., Siedlak, S.L., Galloway, P., Kawai, M., and Cras, P. (1992). Basic fibroblast growth factor binds to filamentous inclusions of neurodegenerative diseases. *Brain Res.* 579:350–352.
- Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E.S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W.G., Lazzarini, A.M.,

- Duvoisin, R.C., Di Iorio, G., Golbe, L.I., and Nussbaum, R.L. (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 276:2045–2047.
- Potter-Perigo, S., Hull, R.L., Tsoi, C., Braun, K.R., Andrikopoulos, S., Teague, J., Verchere, C.B., Kahn, S.E., and Wight, T.N. (2003). Proteoglycans synthesized and secreted by pancreatic islet β -cells bind amylin. *Arch. Biochem. Biophys.* 413:182–190.
- Prusiner, S.B. (1991). Molecular biology of prion diseases. *Science* 252:1515–1522.
- Prusiner, S.B. (1998). Prions. *Proc. Natl. Acad. Sci. USA* 95:13363–13383.
- Prusiner, S.B., and Scott, M.R. (1997). Genetics of prions. *Annu. Rev. Genet.* 28:593–606.
- Rapraeger, A., Jalkanen, M., Endo, E., Koda, J., and Bernfield, M. (1985). The cell surface proteoglycan from mouse mammary epithelial cells bears chondroitin and heparan sulfate glycosaminoglycans. *J. Biol. Chem.* 260:11046–11052.
- Roses, A.D. (1996). Apolipoprotein E alleles as risk factors in Alzheimer's disease. *Annu. Rev. Med.* 47:387–400.
- Roskams, T., de Vos, R., David, G., Van Damme, B., and Desmet, V. (1999). Heparan sulphate proteoglycan expression in human primary liver tumours. *J. Pathol.* 185:290–297.
- Saarela, J., Rehn, M., Oikarinen A., Autio-Harmainen, H., Pihlajaniemi, T. (1998). The short and long forms of type XVIII collagen show clear tissue specificities in their expression and location in basement membrane zones in humans. *Am. J. Pathol.* 153:611–626.
- Sanderson, R.D., Turnbull, J.E., Gallagher, J.T., and Lander, A.D. (1994). Fine structure of heparan sulfate regulates syndecan-1 function and cell behavior. *J. Biol. Chem.* 269:13100–13106.
- Scholefield, Z., Yates, E.A., Wayne, G., Amour, A., McDowell, W., and Turnbull, J.E. (2003). Heparan sulfate regulates amyloid precursor protein processing by BACE1, the Alzheimer's beta-secretase. *J. Cell Biol.* 163:97–107.
- Schonberger, O., Horonchik, L., Gabizon, R., Papy-Garcia, D., Barritault, D., and Taraboulos, A. (2003). Novel heparan mimetics potently inhibit the scrapie prion protein and its endocytosis. *Biochem. Biophys. Res. Commun.* 312:473–479.
- Selkoe, D.J. (1991). The molecular pathology of Alzheimer's disease. *Neuron* 6:487–498.
- Snow, A.D., Willmer, J., and Kisilevsky, R. (1987a). Sulfated glycosaminoglycans in Alzheimer's disease. *Hum. Pathol.* 18:506–510.
- Snow, A.D., Willmer, J., and Kisilevsky, R. (1987b). Sulfated glycosaminoglycans: a common constituent of all amyloids? *Lab. Invest.* 56:120–123.
- Snow, A.D., and Wight, T.N. (1989). Proteoglycans in the pathogenesis of Alzheimer's disease and other amyloidoses. *Neurobiol. Aging* 10:481–497.
- Snow, A.D., Lara, S., Nochlin, D., and Wight, T.N. (1989a). Cationic dyes reveal proteoglycans structurally integrated within the characteristic lesions of Alzheimer's disease. *Acta Neuropathol.* 78:113–123.
- Snow, A.D., Kisilevsky, R., Willmer, J., Prusiner, S.B., and DeArmond, S.J. (1989b). Sulfated glycosaminoglycans in amyloid plaques of prion diseases. *Acta Neuropathol.* 77:337–342.
- Snow, A.D., Mar, H., Nochlin, D., Sekiguchi, R.T., Kimata, K., Koike, Y., and Wight, T.N. (1990a). Early accumulation of heparan sulfate in neurons and in the beta-amyloid protein containing lesions of Alzheimer's disease and Down's syndrome. *Am. J. Pathol.* 137:1253–1270.
- Snow, A.D., Wight, T.N., Nochlin, D., Koike, Y., Kimata, K., DeArmond, S.J., and Prusiner, S.B. (1990b). Immunolocalization of heparan sulfate proteoglycans to the prion protein amyloid plaques of Gerstmann-Straussler syndrome, Creutzfeldt-Jakob disease and scrapie. *Lab. Invest.* 63:601–611.
- Snow, A.D., Bramson, R., Mar, H., Wight, T.N., and Kisilevsky, R. (1991). A temporal and ultrastructural relationship between heparan sulfate proteoglycans and AA amyloid in experimental amyloidosis. *J. Histochem. Cytochem.* 39:1321–1330.
- Snow, A.D., Henderson, M., Nochlin, D., Kresse, H., and Wight, T.N. (1992). Peripheral distribution of dermatan sulfate proteoglycan (decorin) in amyloid-containing plaques and their presence in neurofibrillary tangles of Alzheimer's disease. *J. Histochem. Cytochem.* 40:105–113.
- Snow, A.D., Sekiguchi, R., Nochlin, D., Fraser, P., Kimata, K., Mizutani, A., Arai, M., Schreier, W.A., and Morgan, D.G. (1994). An important role of heparan sulfate proteoglycan (perlecan) in a model system for the deposition and persistence of fibrillar A β -amyloid in rat brain. *Neuron* 12:219–234.
- Snow, A.D., Nochlin, D., Sekiguchi, R., and Carlson, S.S. (1996). Identification and immunolocalization of a new class of proteoglycan (keratan sulfate) to the neuritic plaques of Alzheimer's disease. *Exp. Neurol.* 138:305–317.
- Soto, C. (2001). Protein misfolding and disease; protein refolding and therapy. *FEBS Lett.* 498:204–207.
- Soto, C. (2003). Unfolding the role of protein misfolding in neurodegenerative diseases. *Nat. Neurosci. Rev.* 4:49–60.
- Spillantini, M.G., Schmidt, M.L., Lee, V.M., Trojanowski, J.Q., Jakes, R., and Goedert, M. (1997). Alpha-synuclein in Lewy bodies. *Nature* 388:839–840.

- Spillantini, M.G., Tolnay, M., Love, S., and Goedert, M. (1999). Microtubule-associated protein tau, heparan sulphate and alpha-synuclein in several neurodegenerative diseases with dementia. *Acta Neuropathol.* 97:585–594.
- Stenstad, T., Magnus, J.H., and Husby, G. (1994). Characterization of proteoglycans associated with mouse splenic AA amyloidosis. *Biochem. J.* 303:663–670.
- Su, J.H., Cummings, B.H., and Cotman, C.W. (1992). Localization of heparan sulfate glycosaminoglycan and proteoglycan core protein in aged brain and Alzheimer's disease. *Neuroscience* 51:801–813.
- Supattapone, S. (2004). Prion protein conversion in vitro. *J. Mol. Med.*
- Tanzi, R.E., Gusella, J.F., Watkins, P.C., Bruns, G.A.P., St. George-Hyslop, P., van Keuren, M.L., Patterson, D., Pagan, S., Kurnit, D.M., and Neve, R.L. (1987). Amyloid β protein gene: cDNA, mRNA distribution, and genetic linkage near the Alzheimer locus. *Science* 235:880–884.
- Thompson, A.J., and Barrow, C.J. (2002). Protein conformational misfolding and amyloid formation: characteristics of a new class of disorders that include Alzheimer's and prion diseases. *Curr. Med. Chem.* 9:1751–1762.
- Tsen, G., Halfter, W., Kröger, S., and Cole, G.J. (1995). Agrin is a heparan sulfate proteoglycan. *J. Biol. Chem.* 270:3392–3399.
- Ueda, K., Fukushima, H., Masliah, E., Xia, Y., Iwai, A., Yoshimoto, M., Otero, D.A., Kondo, J., Ihara, Y., and Saitoh, T. (1993). Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease. *Proc. Natl. Acad. Sci. USA* 90:11282–11286.
- van Horssen, J., Otte-Holler, I., David, G., Maat-Schieman, M.L.C., van den Heuvel, L.P.W.J., Wesseling, P., de Waal, R.M.W., and Verbeek, M.M. (2001). Heparan sulfate proteoglycan expression in cerebrovascular amyloid β deposits in Alzheimer's disease and hereditary cerebral hemorrhage with amyloidosis (Dutch) brains. *Acta Neuropathol.* 102:604–614.
- van Horssen, J., Wilhekmus, M.M.M., Heljasvaara, R., Pihlajaniemi, T., Wesseling, P., de Waal, R.M.W., and Verbeek, M.M. (2002). Collagen XVIII: a novel heparan sulfate proteoglycan associated with vascular amyloid depositions and senile plaques in Alzheimer's disease brains. *Brain Pathol.* 12:456–462.
- van Horssen, J., Wesseling, P., van den Heuvel, L.P.W.J., de Waal, R.M.W., and Verbeek, M.M. (2003). Heparan sulphate proteoglycans in Alzheimer's disease and amyloid-related disorders. *Lancet Neurol.* 2:482–492.
- Verbeek, M.M., Otte-Holler, I., van den Born, J., van den Heuvel, L.P., David, G., Wesseling, P., and de Waal, R.M. (1999). Agrin is a major heparan sulfate proteoglycan accumulating in Alzheimer's disease brain. *Am. J. Pathol.* 155:2115–2125.
- Williams, A., Lucassen, P.J., Ritchie, D., and Bruce, M. (1997). PrP deposition, microglial activation, and neuronal apoptosis in murine scrapie. *Exp. Neurol.* 14:433–438.
- Winzen, U., Cole, G.J., and Halfter, W. (2003). Agrin is a chimeric proteoglycan with the attachment sites for heparan sulfate/chondroitin sulfate located in two multiple serine–glycine clusters. *J. Biol. Chem.* 278:30106–30114.
- Wood, J.G., Mirra, S.S., Pollock, N.J., and Binder, L.I. (1986). Neurofibrillary tangles of Alzheimer disease share antigenic determinants with the axonal microtubule-associated protein tau. *Proc. Natl. Acad. Sci. USA* 83:4040–4043.
- Yamaguchi, I., Suda, H., Tszuiki, N., Seto, K., Seki, M., Yamaguchi, Y., Hasegawa, K., Takahashi, N., Yamamoto, S., Gejyo, F., and Naiki, H. (2003). Glycosaminoglycan and proteoglycan inhibit the depolymerization of β_2 -microglobulin amyloid fibrils in vitro. *Kidney Int.* 64:1080–1088.
- Yanagishita, M., and Hascall, V.C. (1992). Cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* 267:9451–9454.
- Young, I.D., Willmer, J.P., and Kisilevsky, R. (1989). The ultrastructural localization of sulfated proteoglycans is identical in the amyloids of Alzheimer's disease and AA, AL, senile cardiac and medullary carcinoma-associated amyloidosis. *Acta Neuropathol.* 78:202–209.
- Young, I.D., Ailles, L., Narindrasorasak, S., Tan, R., and Kisilevsky, R. (1992). Localization of the basement membrane heparan sulfate proteoglycan in islet amyloid deposits in type II diabetes mellitus. *Arch. Pathol. Lab. Med.* 116:951–954.
- Zebrower, M., and Kieras, F.J. (1993). Are heparan sulphate (HS) sulphotransferases implicated in the pathogenesis of Alzheimer's disease? *Glycobiology* 3:3–5.
- Zebrower, M., Beeber, C., and Kieras, F.J. (1992). Characterization of proteoglycans in Alzheimer's disease fibroblasts. *Biochem. Biophys. Res. Commun.* 184:1293–1300.
- Zhu, H., Yu, Y., and Kindy, M.S. (2001). Inhibition of amyloidosis using low molecular weight heparins. *Mol. Med.* 7:517–522.

Apolipoproteins in Different Amyloidoses

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1. Abstract

Formation of amyloid fibrils from an amyloidogenic precursor peptide is a stochastic process. Initially, conditions do not favor aggregation, and this period corresponds to the lag phase that precedes the possible formation of fibrils. This lag phase can be overcome by the amyloidogenic peptide reaching a critically high concentration, or via binding with apolipoproteins or other chaperone proteins. Apolipoproteins interact with numerous amyloidogenic peptides in diverse systemic and organ limited amyloidoses because of their natural tendency to bind hydrophobic domains present on these peptides. Apolipoproteins appear to play an essential role in the kinetics of amyloid deposition. Apolipoprotein E (apo E), which has been found to be involved in the majority of amyloidoses, helps to overcome the initially unfavorable kinetic barrier of amyloid fibril formation, and typically shows isoform-specific effects that influence the epidemiology, clinical course, and pathological aspects of disease. The modulatory effect of apolipoproteins may not be evident if either the amyloid-prone protein is present in great excess or carries a mutation rendering it extremely amyloidogenic. Under these conditions amyloid deposition can occur without the help of chaperones. The exact binding site of amyloidogenic peptides on apo E and other apolipoproteins is unclear, and it remains unknown if it is a linear or a conformational epitope. Mapping this site could provide a means to clarify the mechanism of interaction between apolipoproteins and their peptide ligands, as well as allowing the generation of peptidomimetic compounds that block this interaction in an effort to develop successful treatment approaches. It appears that apo E has one universal site interacting with various amyloidogenic peptides regardless of their sequence. Targeting the pathological chaperones of amyloidoses such as apo E appears to be an attractive approach from a pharmacokinetic point of view, because the concentration of these molecules in amyloid deposits is usually 100 to 200 times lower than the concentration of the actual fibril-forming peptide (Ma *et al.*, 1994; Wisniewski *et al.*, 1994a). In addition, blocking this interaction between apo E and amyloidogenic proteins is unlikely to be associated with any significant toxicity, making this a significant therapeutic target.

2. Introduction

Amyloidoses are disorders of protein conformation, in which low molecular weight proteins or protein fragments, that are soluble and degradable under physiological conditions, form insoluble amyloid fibrils and accumulate in diverse tissues and organs (Merlini and Bellotti, 2003; Selkoe, 2003). Proteins or protein fragments involved in the various amyloidoses have different amino acid sequences and sizes, but despite this biochemical diversity, all of them are capable of adopting a

common antiparallel β -sheet secondary structure that is a prerequisite for fibril formation (Merlini and Bellotti, 2003). After assuming a β -sheet pleated pro-amyloidogenic conformation these proteins can assemble into fibrils by forming hydrogen bonds between their exposed hydrophobic domains. This is a stochastic process that depends on the presence of a critical concentration of the amyloidogenic peptide in a pro-aggregation conformation in a given time and place. Therefore, amyloidoses arise and develop in the setting of an elevated concentration of specific amyloid precursor proteins or protein fragments in physiological fluids or organs, which in turn, can be a result of either their increased production, or impaired clearance, or both.

Amyloid deposits are associated with the invariable presence of certain amyloid-associated proteins such as the amyloid-P component (Coria *et al.*, 1988), sulfated proteoglycans (Snow *et al.*, 1987, 1989, 1990), α 1-antichymotrypsin, complement factors (Johnson *et al.*, 2002), and various apolipoproteins: apolipoprotein (apo) A1 (Wisniewski *et al.*, 1995a), B (Namba *et al.*, 1992), apolipoprotein E (apo E) (Namba *et al.*, 1991; Wisniewski and Frangione, 1992), and J (Choi-Miura *et al.*, 1992). Several lines of evidence have demonstrated that apolipoproteins are more than just innocent bystanders trapped in amyloid fibrils due to their affinity for hydrophobic domains, but they play a crucial role in the process of amyloid fibril formation and deposition in tissue. Furthermore, fragments of apolipoproteins can form amyloid fibrils themselves (Wisniewski *et al.*, 1995a, 1995b). Apolipoproteins are a distinct family of proteins playing a pivotal role in proper trafficking of cholesterol in blood stream (Mahley, 1988) and in the nervous system. They contain two major functional domains: the hydrophobic domain, which is used to take up lipids, and the receptor domain, allowing binding to a specific receptor to deliver lipids to their proper tissue destination (**Figure 6-1A; see color insert**). Apolipoproteins possess certain structural plasticity, allowing interactions with hydrophobic ligands. They undergo major structural changes of unfolding and refolding when lipids are released or taken up, respectively. In conditions favoring amyloidoses, where high concentration of amyloidogenic peptides with exposed hydrophobic domains are present, different apolipoproteins can bind and modulate aggregation/fibril formation in a variety of ways including: (1) transport of the amyloidogenic peptides, (2) propagation of the β -sheet structure and facilitation of fibril formation, or conversely, (3) binding to the amyloidogenic peptides and inhibiting their aggregation into fibrils. In addition, hydrophobic fragments of some apolipoproteins may form amyloid fibrils themselves. They can be codeposited together with fibrils formed by the primary amyloidogenic peptide, or in rare cases they may become the major component of the amyloid deposits. These complex interactions between apolipoproteins and amyloidogenic peptides in different amyloidoses can have a major impact on the clinical course of these diseases. The prototypical example demonstrating the multiple ways different apolipoproteins can be involved in amyloid deposition is Alzheimer's disease (AD). Alzheimer's disease is the most common cause of dementia, affecting today over 4 million people in the United States alone and about 14 million worldwide. It is associated with deposition of β -amyloid ($A\beta$), a 39–43-amino acid long peptide in the form of plaques in the brain parenchyma and in the walls of meningocerebral arteries. Ninety-five percent of all AD cases have an onset after the age of 65, while 5% are early onset and have an autosomal dominant inheritance pattern. The major modifying genetic risk factor for sporadic AD identified so far is inheritance of different apo E isoforms (Strittmatter *et al.*, 1993a). Subjects who inherit the apo E4 isoform (**Figure 6-1B; see color insert**), demonstrate in an allele dose-dependent manner, increased likelihood for disease occurrence, lower age of onset, and increased density of amyloid deposits in the brain (Mayeux *et al.*, 1993; Saunders *et al.*, 1993b, 1993c; Maestre *et al.*, 1995; Relkin *et al.*, 1996). In this chapter we will review the roles of different apolipoproteins in the pathological mechanism of various amyloidoses and their modulatory effect on the clinical presentation of these diseases.

3. Molecular Characteristic of Apolipoproteins Involved in Amyloidoses

3.1. Apolipoprotein A

Apolipoproteins A constitutes a family of several apolipoproteins termed AI, AII, and AIII. Apolipoprotein AI (apo AI) is a 28-kDa nonglycosylated protein, which is a major protein component of serum high-density lipoproteins (HDL). Therefore, apo AI is also called a major antiatherosclerotic apolipoprotein (Kostner, 1989; Rader, 2003). In the central nervous system (CNS) apo AI is secreted by CNS astrocytes, and like apo E forms CNS HDLs, which are used to redistribute and deliver lipids to neurons.

3.2. Apolipoprotein E

Apo E is a 34-kDa glycosylated protein encoded on chromosome 19q13.2 (**Figure 6-1A and B; see color insert**).

The N-terminal domain of apo E contains four class G amphipathic helices, whereas its C-terminal domain has class A amphipathic helices. These domains are connected via a thrombin cleavable unstructured region. Residues 192–299 of the C-terminal domain constitute the apo E major lipid binding region, whereas residues 136–160 form the receptor binding region (Segrest *et al.*, 1994; Weisgraber, 1994). Apo E consists of 299 amino acids with variations at positions 112 and 158 producing three isoforms E2 (cysteine/cysteine), E3 (cysteine/arginine), and E4 (arginine/arginine). These minor sequence differences are associated with distinct tertiary structures and affinities to lipoprotein receptors (Dong *et al.*, 1994; Mahley *et al.*, 1996). Apo E4, but not E2 or E3, shows a strong interaction between N- and C-terminal domains, which is mediated by an ionic interaction between arginine-61 and glutamic acid 255. E3 and E4 isoforms containing an arginine at position 158 have similar binding to apo E receptors, which is better than the E2 isoform that has a cysteine at position 158. As a result, apo E2 isoform carriers are at risk for type III hyperlipoproteinemia (Weisgraber, 1994). The frequency of E2, E3, and E4 alleles in the general population is as follows: 9, 77, and 14%, respectively.

Apo E is produced by hepatocytes in the liver, Schwann cells in the peripheral nervous system (PNS), and by astrocytes in the CNS (Pitas *et al.*, 1987). The major function of the liver-born apo E is transportation of lipids among various tissues. Apo E is a constituent of chylomicrons, very low-density lipoproteins (VLDLs), and a subclass of HDL but not low-density lipoproteins (LDL). It functions as a ligand for several lipoprotein receptors (Mahley, 1988). Apo E knock-out (KO) transgenic (tg) mice demonstrate severe hyperlipidemia (Kostner, 1989; Rader, 2003). In the PNS and in the CNS apo E plays a critical role in mobilization and redistribution of cholesterol during growth, synapse formation, and following injury (Boyles *et al.*, 1989). HDLs are major lipid-carrying particles in the CNS. Unlike serum HDL, most CNS HDLs contain apo E as a main lipoprotein (Pitas *et al.*, 1987; Boyles *et al.*, 1989). Apo AI also forms CNS HDLs, but these two apolipoproteins exist largely on different particles (Boyles *et al.*, 1989). Apo E receptors are located mainly on neurons and reactive astrocytes. Apo E delivers cholesterol necessary for neuronal repair, neurite branching, extension, and synaptic plasticity (Nathan *et al.*, 1994; Masliah *et al.*, 1997; Ji *et al.*, 2003).

3.3. Apolipoprotein J

Apolipoprotein J (apo J) is also known as clusterin or SP-40-40. It is encoded by a gene located on chromosome 8 (Dietzsch *et al.*, 1992). Apo J is a disulfide-linked heterodimeric glycoprotein

composed of two 40 kDa subunits termed as α - and β -chains, which are derived from a single mRNA molecule by cleavage between residues arginine-205 and serine-206 (Tsuruta *et al.*, 1990; Kirszbaum *et al.*, 1992). Unlike apo E or apo AI, the role of apo J is not mainly limited to lipid transport (Koudinov *et al.*, 1994), but also includes secretion (Hartmann *et al.*, 1991), membrane recycling, spermatogenesis (Griswold *et al.*, 1986; Choi *et al.*, 1990b, 1990c), and modulation of the complement pathway (Choi *et al.*, 1990a; Kirszbaum *et al.*, 1992). Apo J is expressed in almost all tissues including the CNS, where its messenger RNA is specifically found in neurons and astrocytes (Danik *et al.*, 1991). Apo J is capable of crossing the blood–brain barrier (BBB) using a specific receptor/transport mechanism that may have an important effect on A β trafficking and deposition in AD (see below).

4. Role of Apolipoproteins in Pathological Mechanism of AD Amyloidosis

Alzheimer's disease is an incurable neurodegenerative illness associated with neuronal and synaptic loss, linked to the presence of neurofibrillary tangles and A β deposition in the form of plaques in the gray matter of the brain and in the walls of meningeocephalic arteries (Ingelsson *et al.*, 2004). There are over 4 million people suffering for AD in the United States today, and 95% of them have a sporadic form of this disease. According to the A β cascade theory, a disturbance of A β homeostasis in the brain is central to the pathological mechanism of AD (Selkoe, 2000). A β is a 39–43-amino acid peptide, which is a derivative of larger 750-amino acid transmembrane protein—the amyloid precursor protein (APP). The amyloid β precursor protein, which is abundantly expressed at the synaptic junction of the CNS neurons, can be cleaved by a number of secretases. Cleavage by β and γ secretases in the amyloidogenic pathway results in production of the A β peptide. Alternatively, APP can be processed in the nonamyloidogenic pathway by α and γ secretases (Gandy *et al.*, 1994; De Strooper, 2003). Under normal conditions soluble A β (sA β) is locally degraded or removed from the brain by receptor-mediated transport across the BBB (Gherzi-Egea *et al.*, 1996; Zlokovic *et al.*, 2000; Monro *et al.*, 2002). Accumulation of A β in AD brains is associated with increased production (Fukumoto *et al.*, 2002, 2004) and/or impaired clearance, which are observed both with advancing age and in sporadic AD (Shibata *et al.*, 2000). In addition, sA β , which is circulating in the blood stream, may cross the BBB and co-deposit on existing plaques (Zlokovic *et al.*, 1993; Wengenack *et al.*, 2000). The remaining 5% of inherited AD is associated with mutations that lead to either increased production of total A β or the more fibrillogenic A β 1–42 species (Selkoe, 1995, 1997). The association of apo E isotypes on the incidence of sporadic AD was demonstrated for the first time by Roses and colleagues, who studied a relatively small cohort of families with a high incidence of sporadic AD and showed that the apo E4 isoform, in an allele dose-dependent manner, is associated with an increased occurrence of sporadic AD (Corder *et al.*, 1993). This initial observation has been confirmed by numerous studies performed in ethnically different populations, showing that the risk for AD is increased among E4 heterozygotes by three- to fourfold and up to 14-fold for homozygotes, with an earlier age of onset lower on average by 5 and 10 years, for hetero- and homozygotes compared to non-E4 carriers, respectively (Mayeux *et al.*, 1993; Saunders *et al.*, 1993b, 1993c; Maestre *et al.*, 1995; Relkin *et al.*, 1996). Similar to sporadic AD, a relationship between apo E4 allele and an earlier onset of AD dementia (Schupf *et al.*, 1996; Schupf, 2002) and, with a faster rate of cognitive decline (Del Bo *et al.*, 1997, 2003) was demonstrated in subjects with Down's syndrome. On the other hand, 50% of sporadic AD patients do not have an apo E4 allele, and many who are either E4 hetero- or homozygotes do not develop AD. Therefore, the presence of the apo E4 isoform is not a prerequisite for disease occurrence, but rather has a modulatory risk factor. This striking epidemiological associa-

tion led to many studies into the possible mechanisms of apo E's involvement in AD, as well as for further studies to determine whether other lipoproteins are involved. It has been demonstrated that apos A1, E, and J can bind with high affinity directly to A β . Apo E can effectively promote the conversion of soluble A β (sA β) into fibrillar A β with an isoform-specific effect analogous to that observed for occurrence of AD in epidemiological studies, apo E₄ being the most pro-fibrillogenic. (Wisniewski and Frangione, 1992; Sanan *et al.*, 1994; Wisniewski *et al.*, 1994a). Furthermore, neuropathological studies showed that the inheritance of the E4 allele correlates with a greater density of senile plaques in the cerebral cortex and increased deposition of A β in blood vessels (Rebeck *et al.*, 1993; Schmechel *et al.*, 1993; Yamaguchi *et al.*, 2001). The following sections will discuss the involvement of apos E, J, and A1 in various aspects of A β metabolism, trafficking, and deposition in AD.

4.1. Effect of Apo E on A β Fibrillization and Deposition

The secondary structure of the sA β peptide, released from APP, is dominated mainly by an α -helix and random coil conformation (Wisniewski *et al.*, 1994b). sA β , as almost every other amyloidogenic peptide, needs to first undergo a conformational transformation into a β -sheet pleated structure to assemble into fibrils. This is a stochastic and long-lasting process. Experiments with A β homologous synthetic peptides demonstrate amyloid fibril formation in solution at a critical concentration (Kirschner *et al.*, 1987; Hilbich *et al.*, 1991; Wisniewski *et al.*, 1991; Barrow *et al.*, 1992; Burdick *et al.*, 1992; Stine *et al.*, 2003). This process can be effectively accelerated by the addition of apo E in a molar ratio of A β to apo E of 100–200:1 (**Figure 6-2; see color insert**) (Ma *et al.*, 1994; Wisniewski *et al.*, 1994a). *In vitro* all apo E isoforms can promote the β -sheet content of A β peptides, promoting fibril formation (Castaño *et al.*, 1995; Golabek *et al.*, 1996), with apo E4 being the most efficient (Ma *et al.*, 1994; Wisniewski *et al.*, 1994a). The effect of apo E on A β fibril formation appears to work through stabilization of the A β fibril structure (Figure 6-3). Apo E binds hydrophobically to A β in a molar ratio of 1:1, forming SDS-page insoluble complexes (**Figure 6-2C**;

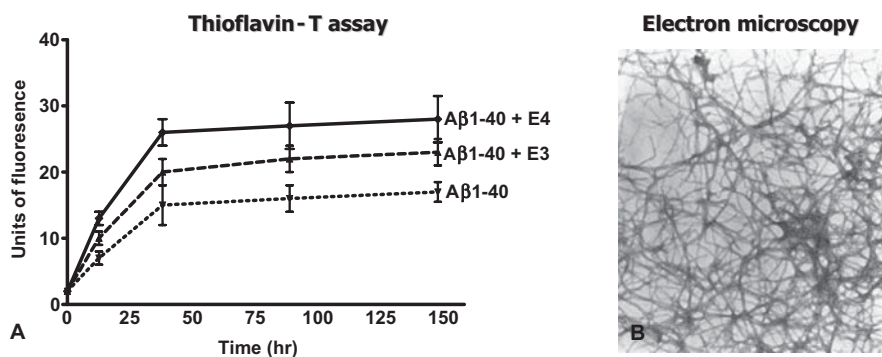


Figure 6-3. A classical experiment demonstrating pro-fibrillogenic activity of apoE. (A) Synthetic peptide homologous to A β 1–40 sequence incubated in a concentration of 100 μ M in 100 mM Tris buffer (pH 7.2). Adding apo E in proportion apo E to A β 1:100 increases the amount of fibrils formed over time as detected by the Thioflavin-T fluorimetric assay. Isoform specific effect can be demonstrated. There are more fibrils formed in the presence of apo E4 than apo E3. (B) Fibrils formed by synthetic apo E peptide in the presence of apo E visualized by electron microscopy negative staining.

see color insert) (Wisniewski *et al.*, 1993b; Naslund *et al.*, 1995; Golabek *et al.*, 2000). The affinity of binding ranges between a K_D of 10 and 20 nM, and is largely dependent on the conformational state of A β with a higher affinity toward fibrillar more β -sheet pleated A β than compared with soluble A β (Strittmatter *et al.*, 1993a; Golabek *et al.*, 1995, 1996; Shuvaev and Siest, 1996). Recent studies using solid-state NMR techniques brought new and valuable information on the mechanism of A β fibril assembly and the possible role of apo E. A β peptide-forming fibrils assumes a U-shape form due to a β -turn involving residues 25–29. This β -turn is critical in bringing into contact two hydrophobic β -strand domains involving residues 12–24 and 29–40/42 (Petkova *et al.*, 2002), allowing for nucleation and fibril formation related to hydrophobic interactions between A β monomers (Antzutkin *et al.*, 2003; Petkova *et al.*, 2004). Apo E binds to residues of 12–28 of A β (Strittmatter *et al.*, 1993a, 1993b; Ma *et al.*, 1996), which is the β -turn region, and therefore, it may stabilize the β -sheet pleated pro-fibrillar A β conformation and accelerate fibril formation. These observations have led to the proposal that apo E may function as a “pathological chaperone” of A β in the pathogenesis of AD. This term defines a protein that interacts with A β and helps to promote a conformational shift from sA β into fibrillar A β , and furthermore stabilize its abnormal conformation, associated with disease. This contrasts with the normal physiological chaperone proteins, which act to induce and preserve the normal, native conformation (Beissinger and Buchner, 1998).

Involvement of apo E appears to be critical for A β deposition *in vivo*. Apo E gene KO in APPV717F AD tg mice, which normally develop A β deposits, results in a significant reduction of A β load and the virtual absence of fibrillar A β (Figure 6-4; see color insert) (Bales *et al.*, 1997, 1999). Amyloid precursor protein tg mice expressing one copy of apo E show an intermediate level of pathology (Bales *et al.*, 1999). Immunohistochemical studies have identified the presence of apo E in AD plaque lesions (Namba *et al.*, 1991; Wisniewski and Frangione, 1992), but only in those containing fibrillar A β deposits, which are associated with local neurotoxicity, as indicated by the presence of dystrophic neuritis and focal neuronal loss (Figure 6-5; see color insert) (Mufson *et al.*, 1994; Wisniewski *et al.*, 1995b). In contrast, preamyloid aggregates that accumulate A β peptides but do not contain A β fibrils, are not associated with local neurotoxicity and are apo E negative (Gallo *et al.*, 1994; Wisniewski *et al.*, 1998). These observations demonstrate that apo E, through the promotion of A β fibril formation, leads to A β sequestration within the brain and accumulation in the form of plaques or in the walls of meningocephalic vessels (Frangione *et al.*, 1994; Castaño *et al.*, 1995), initiating a neurodegenerative cascade (Thomas *et al.*, 1996; Yan *et al.*, 1996; Holtzman *et al.*, 2000b; Lewis *et al.*, 2001; Stine *et al.*, 2003). Other proteins, which have been isolated from A β plaques, can also propagate the *in vitro* assembly of A β homologous peptides into fibrils and to some extent have also been shown to do this *in vivo*. Examples of these include α 1-antichymotrypsin (ACT) (Ma *et al.*, 1994; Potter *et al.*, 2001) or the C1q complement factor (Johnson *et al.*, 2002; Boyett *et al.*, 2003). The term pathological chaperones can be extended to these proteins; however, their overall role appears to be less significant compared to apo E.

The *in vitro* studies demonstrated that all apo E isoforms can act effectively as pathological chaperones of A β , with apo E4 being the most efficient (Wisniewski *et al.*, 1994a). This observation is consistent with epidemiological observations, and provides one explanation why the apo E4 isoform increases the likelihood of disease occurrence and decreases the age of onset, but is not absolutely required for disease occurrence. Hence, under conditions facilitating increased production or decreased clearance of A β , the impact of apo E's chaperoning effect becomes evident. The association between differences in the molecular structure of the three apo E isoforms and their effect on A β fibril formation are not fully understood. *In vitro* experiments using A β homologous peptides demonstrate a promoting effect of apo E isoforms in decreasing order of E4:E3:E2, which is compatible with conclusions drawn from epidemiological observations (Saunders *et al.*, 1993c; Maestre *et al.*,

1995; Relkin *et al.*, 1996). Consistent results were obtained using double tg mice coexpressing mutated human APP and various forms of human apo E on a murine apo E KO background. Apo E isoforms in the following order: E4, E3, and E2 were associated with decreased A β load.

The relationship between the various apo E isoforms and their effect on A β fibrilization could be better understood knowing the structure of the A β binding domain on apo E. Initial data, based on the 10-kDa C-terminal fragment isolated from plaque deposits (Wisniewski *et al.*, 1995b), suggested that A β binds to residues 244–272 of apo E (Strittmatter *et al.*, 1993b; Pillot *et al.*, 1997). In contrast, more recent studies with thrombin-cleaved apo E fragments demonstrated that the N-terminal domain binds A β with a K_D = 11 nM (Golabek *et al.*, 2000), which is 10 times higher than the affinity of the C-terminal domain, and is comparable to the affinity of undigested apo E (Golabek *et al.*, 1996; Shuvaev and Siest, 1996; Tokuda *et al.*, 2000). Furthermore, the N-terminal domain forms sodium dodecyl sulfate (SDS)-resistant complexes with A β , whereas the C-terminal domain A β binding can be easily disrupted by SDS (Golabek *et al.*, 2000). Hence, there appears to be more than one A β binding site on apo E, each with different affinities.

4.2. Role of Apolipoproteins in A β Trafficking Across the BBB, in the Serum and in the CSF

Soluble A β is present in all physiological body fluids including the cerebrospinal fluid, serum, and urine (Ghiso *et al.*, 1993, 1997), whereas fibrillization and deposition of A β takes place only in the brain and in the walls of cerebrovasculature. Soluble A β is present in body fluids as a complex with other proteins such as apo J, which is a part of HDL particles (Koudinov *et al.*, 1994). Unlike apo E, apo J has a five times higher affinity to soluble A β compared to aggregated A β (Matsubara *et al.*, 1995). Biochemical data indicate that apo J is a major ligand for sA β in plasma and CSF, and that apo J may serve as a carrier protein in these body fluids preventing it from self-aggregation. Therefore, apo J has a fibril suppression property (Ghiso *et al.*, 1993; Wisniewski *et al.*, 1993a; Matsubara *et al.*, 1995).

sA β circulating in the blood can penetrate the BBB as a free peptide (Poduslo *et al.*, 1999; Wengenack *et al.*, 2000) or complexed with apolipoproteins, which constitute the bulk of transport across BBB (Zlokovic *et al.*, 1993, 1994). Both forms of transport are based on specific receptor mechanisms. Transport of free sA β occurs via the receptor for advanced glycation end products (RAGE) (Yan *et al.*, 1996; Deane *et al.*, 2003), and the scavenger receptor type A (SR-A) (Paresse *et al.*, 1996). They facilitate endothelial endocytosis and transcytosis of A β that is initiated at the luminal site of the BBB (Mackic *et al.*, 1998; Mackic *et al.*, 2002). The rate of the serum-to-brain transport occurs severalfold more efficiently when sA β is complexed with apo J, compared to apo E (Zlokovic *et al.*, 1993, 1994). This is mediated by the LDL receptor-related protein 2 (LRP-2) (Zlokovic *et al.*, 1996). Therefore, apo J may act as a double-edged sword on one hand preventing A β from self-aggregation in body fluids, but on the other hand, facilitating the passage of A β across the BBB into the brain, where it may deposit.

Removal of A β across the BBB from the CNS is mediated by LDL receptor-related protein 1 (LRP-1) and is facilitated by both apo E and apo J (Zlokovic, 1996; Shibata *et al.*, 2000). In mice with apo E KO removal of radiolabeled A β injected into the brain is impaired compared with wild-type (wt) mice. Also, tg mice expressing a human apo E4 isoform on murine apo E KO have worse A β clearance than those expressing E2 and E3 (Ji *et al.*, 2001). This dual role of apo E in A β clearance and deposition is likely dependent on the concentration of CNS A β and that of other A β binding proteins. Reduced A β load in APP/apoE KO tg mice demonstrate that the role of apo E as a patho-

logical chaperone outweighs its role in A β clearance (Bales *et al.*, 1997, 1999; Holtzman *et al.*, 1999, 2000a). This becomes especially important, with advancing age when LRP-1 is downregulated and A β clearance becomes inefficient (Shibata *et al.*, 2000).

4.3. Apolipoproteins and Neuronal Pathology in AD

Apo E has been implicated in the process of promoting intracellular accumulation of A β (Yan *et al.*, 1997; Tomiyama *et al.*, 1999). The intracellular presence of A β has been demonstrated in patients with sporadic AD (Gouras *et al.*, 2000a, 2000b), DS subjects (Mori *et al.*, 2002), and in AD tg mice (Wirhth *et al.*, 2001) before initial extracellular A β deposits become **detectable**. Neurons express various apo E receptors including the LRP receptor (Rebeck *et al.*, 1993). In conditions where local brain concentrations of apo E are increased, such as in sporadic AD, apo E may become a “Trojan horse” by binding sA β and facilitating the entry of apo E/A β /lipid complexes into neurons (Rebeck *et al.*, 1993; Tomiyama *et al.*, 1999). Because increased neurite sprouting and synaptic plasticity occurs in the AD brain to compensate for neuronal dropout and synaptic loss, the need for lipid intake is increased, creating a greater likelihood of neuronal A β uptake via the apo E/LRP mechanism and initiating a vicious cycle. Apo E/HDL complexes become delipidated, and are cleared by the endosomal/lysosomal pathway. The acidic milieu of late endomes and lysosomes constitute ideal conditions for A β fibrilization, because lowering the pH promotes fibril assembly (Stine *et al.*, 2003), especially in the presence of apo E. In an experiment using cultured human neuroblastoma cells, most endocytosed A β peptides were degraded, but some peptide was found to be accumulated within the cells (Ida *et al.*, 1996). The same has been demonstrated for vascular smooth muscle cells where formation of more stable deposits is associated with the apo E4 isoform (Mazur-Kolecka *et al.*, 1995). In AD brain intracellular accumulation of A β correlates with apo E uptake and DNA fragmentation (LaFerla *et al.*, 1997). Furthermore, the formation of A β oligomers was recently demonstrated within processes and synapses of cultured Tg2576 APP tg mice neurons, and in brains of these tg mice and in humans with AD (Takahashi *et al.*, 2004). Synaptic dysfunction and loss is the first detectable neuropathological hallmark of AD strongly associated with cognitive impairment (Ingelsson *et al.*, 2004).

4.4. Apolipoproteins and Cerebral Amyloid Angiopathy

Cerebral amyloid angiopathy (CAA) is a condition resulting from deposition of amyloidogenic peptides in the walls of meningeocerebral vessels (for review, see Revesz *et al.*, 2003). Except for rare cases of familial CAA related to mutations of transthyretin, cystatin C (Levy *et al.*, 1989), gelsolin (Levy *et al.*, 1990b), and British or Danish dementia precursor protein (Ghiso *et al.*, 2000), the most frequent cause of CAA is accumulation of A β (Ghiso *et al.*, 1994). Deposition of A β in the meningeocerebral vasculature is found in 46% of individuals over the age of 70 years (Revesz *et al.*, 2002), regardless of whether they have AD or not. In patients fulfilling criteria for the neuropathological diagnosis of AD, CAA is found in 80% of cases, and in approximately 25% is moderate to severe. There is also a distinct group of elderly individuals with an extensive amount of CAA but with modest AD changes in the brain parenchyma. They frequently suffer from cerebral lobar hemorrhages due to weakening of vascular walls by amyloid deposits and associated focal inflammation (Revesz *et al.*, 2002). The overlap between the biology of sporadic CAA and AD suggest that these two conditions share common risk factors. Unlike parenchymal amyloid plaques, vascular A β amyloid contains mainly A β 1–40 species, with a relatively lower amount of A β 1–42. Immunoreactivity of both apo E and apo J was detected in vascular deposits (Namba *et al.*, 1991; Verbeek *et al.*, 1998). It is therefore believed that following initial seeding with A β 1–42 in the vascular wall, subsequent expansion of the

deposits occurs by incorporation of the less fibrillogenic A β 1–40, with the presence of the apo E4 isoform promoting these vessel amyloid lesions (Van Dorpe *et al.*, 2000; Revesz *et al.*, 2003). Like parenchymal deposits, the apo E4 isoform in the allele dose-dependent manner is associated with an increased likelihood of CAA. This association between apo E4 and CAA is irrespective of the presence or absence of parenchymal AD-related pathology (Kalaria and Premkumar, 1995; Greenberg *et al.*, 1996; Kalaria *et al.*, 1996; Premkumar *et al.*, 1996). Those AD patients with a moderate to severe degree of CAA, show an increased incidence of cerebral hemorrhages and ischemic lesions superimposed on AD pathology (Ellis *et al.*, 1996). Therefore, the apo E4 isoform indirectly contributes to a cognitive decline in these patients by promoting vascular pathology. Surprisingly, the apo E2 isoform, which seems to be have the least effect on A β deposition promotion (Thal *et al.*, 2002), has been associated with an increased risk of hemorrhage in CAA patients (Alonzo *et al.*, 1998). However, neuropathological studies of patients with the apo E2 allele and CAA-related hemorrhage have shown increased degeneration of vascular walls, which makes them more prone to rupture, without increased amyloid deposition (Alonzo *et al.*, 1998; Greenberg *et al.*, 1998), suggesting that apo E2 is influencing the incidence of hemorrhage through nonamyloid-related pathways.

Relatively little work has investigated the role of apo E and other apolipoproteins in familial amyloidoses. Severe CAA changes were found to be associated with some of mutations of presenilin (PS) 1 and 2 responsible for young onset, familial AD phenotype (Nochlin *et al.*, 1998; Mann *et al.*, 2001; Steiner *et al.*, 2001; Takao *et al.*, 2002) and with APP mutations that result in amino acid substitution within residues 21 and 23 of A β . The latter missense mutations are typically associated with profound CAA changes, with members of affected kindreds typically coming to medical attention with hemorrhagic and ischemic strokes (Levy *et al.*, 1990a; Revesz *et al.*, 2003). A lack of a modulatory effect by apo E isoforms in these conditions has been reported (Bornebroek *et al.*, 1997), and it was concluded that the chaperoning effect of apolipoproteins in these conditions may not be as critical as it is in sporadic AD and CAA. The PS mutations lead to increased production of the highly fibrillogenic A β 1–42, while the mutated A β with substitutions in positions 21 or 23 is also highly fibrillogenic. These peptides show an overwhelming propensity for self-aggregation; therefore, any effect of chaperone proteins may be minimal (Castano *et al.*, 1995b). The presence of apoe E and J was also detected in vascular amyloid in familial British and Danish dementias patients, although the clinical impact of apo E isoforms in these conditions is unknown (Revesz *et al.*, 2002; Rostagno *et al.*, 2002).

5. Apolipoproteins in Prion Diseases

Prion diseases are a group of invariably fatal neurodegenerative diseases associated with the conformational transformation of the physiological prion protein PrP^C (C-cellular) into a toxic and infectious conformer PrP^{Sc} (Sc- scrapie), which acquires the ability to multiply by binding to PrP^C and using it as a template for its own replication (DeArmond *et al.*, 2002). The conformational transformation of PrP^C into PrP^{Sc} is associated with a significant increase in β -sheet content (Aucouturier *et al.*, 1999), and PrP^{Sc} is capable of self-assembly into dimers and oligomers (Prusiner *et al.*, 1983a, 1983b; Knaus *et al.*, 2001). Oligomeric forms of PrP^{Sc} exists as amyloid-like rods in the extraneuronal space (Prusiner *et al.*, 1983c), although formation of true amyloid plaques composed of fragments of PrP protein in the brain parenchyma is a constant feature of only some prion diseases such as: Gerstmann-Sträussler-Scheinker syndrome, variant Creutzfeldt-Jakob (vCJD) disease, and kuru. They are also present in 10% of sporadic CJD (sCJD) cases (DeArmond *et al.*, 2002). Although amyloid deposits associated with prion diseases and amyloid deposits in AD are composed of different peptides, they have a similar ultrastructure and share common physicochemical properties (Merz *et al.*, 1983). These similarities between AD and prionoses suggested the possibility that apolipoproteins

may be PrP-associated proteins, possibly facilitating the pathological conformational transition. Apo E could play a role in at least two aspects of prion related pathology: (1) formation of oligomeric PrP^{Sc} aggregates in the interneuronal space, and (2) formation of true amyloid fibrils. In fact, the presence of apo E has been detected in amyloid plaques in sporadic CJD (Namba *et al.*, 1991), and apo E expression was found to be upregulated in prion-infected animals (Diedrich *et al.*, 1991). Furthermore, synthetic peptides homologous to amyloidogenic PrP protein fragments (e.g., encompassing PrP residues 109–122 or PrP residues 109–141) has been found to bind to apo E and form strong hydrophobic SDS-resistant complexes similar to A β peptides (Baumann *et al.*, 2000). Also, similar to A β peptides, apo E influences the content of β -sheet conformation of these PrP synthetic peptides *in vitro* and actively promotes their aggregation into fibrils (Baumann *et al.*, 2000).

In contrast to AD, however, epidemiological analysis of apo E isoforms frequency among patients with various forms of prion diseases does not uniformly support the association between apo E4 and increased risk for prionoses. The initial study analyzing only 10 cases demonstrated the same frequency of apo E4 allele in affected subjects as in the general population (Saunders *et al.*, 1993a). A subsequent study analyzing a larger cohort of 61 cases including sporadic and familial CJD showed an increased E4 allele frequency among affected individuals (Amouyel *et al.*, 1994). No confirmation of this study was found in several of the reports studying sporadic, iatrogenic, and familial CJD, fatal familial insomnia, and Gerstmann-Sträussler-Scheinker syndrome (Nakagawa *et al.*, 1995; Salvatore *et al.*, 1995; Zerr *et al.*, 1996; Chapman *et al.*, 1998), while a more recent study in 126 sporadic CJD patients suggested that the apo E4 allele is an independent risk factor for developing CJD (Van Everbroeck *et al.*, 2001). Interpretation of these epidemiological studies is complicated by the relatively low number of cases reported. One study assessing the incubation period of prion infection in apo E KO tg mice did not support a role of apo E in scrapie infection (Tatzelt *et al.*, 1996); however, it is possible that the role of apo E in prion infection may show strain/species differences.

6. Apolipoproteins in Other Amyloidoses

Several lines of evidence tie apolipoproteins, especially apo E, to the process of amyloid deposition in various systemic and organ limited amyloidoses. Apo E has been copurified from amyloid deposits in various conditions including primary (amyloid L-light chains of immunoglobulins) and secondary (amyloid A; 76-residue proteolytic fragment of serum protein A) systemic amyloidoses (Castano *et al.*, 1995a; Cui *et al.*, 1998), primary and secondary cutaneous amyloidoses (Chang *et al.*, 2001; Furumoto *et al.*, 2002), and isolated atrial amyloidosis (Takahashi *et al.*, 1998). The presence of the apo E4 allele has been determined as a risk factor for the occurrence of amyloid-A amyloidosis in patients with rheumatoid arthritis (Hasegawa *et al.*, 1996) and β_2 -microglobulin amyloidosis in hemodialysis-related amyloidosis (Gejyo *et al.*, 1997). Studies have shown also that apo E *in vitro* binds to synthetic peptides homologous to amyloidogenic fragments of the amyloid A peptide, and gelsolin (Castano *et al.*, 1995a; Soto *et al.*, 1995) deposited in familial Finnish hereditary amyloidosis (Haltia *et al.*, 1990). This interaction modulates the β -sheet content and actively accelerates the rate of fibril formation (Castano *et al.*, 1995a; Soto *et al.*, 1995; Baumann *et al.*, 2000; Fadika and Baumann, 2002). It has been also shown that A β , and amyloidogenic fragments of serum protein-A, gelsolin, and PrP, bind to the same site on apo E; and in competitive binding experiments they may displace each other (Castano *et al.*, 1995a; Soto *et al.*, 1995). The essential role played by apo E in the process of amyloid A deposition was confirmed using apo E KO mice in which amyloid formation was greatly reduced (Kindy and Rader, 1998). A similar relationship could not be demonstrated in tg mice expressing the islet amyloid polypeptide (IAPP), which is a model of pancreatic islet amyloidosis associated with type II diabetes. No difference in amyloid load was found among

three different strains, the IAPP^{+/+}/apo E^{+/+}, IAPP^{+/+}/apo E^{+/-}, IAPP^{+/+}/apo E^{-/-} lines (Vidal *et al.*, 2003), which suggests that apo E does not play a role in this form of organ-limited amyloidosis. An alternative interpretation can be that with the overwhelming IAPP overexpression in this model mice, the presence of apo E is not critical for formation of amyloid deposits.

7. Apolipoproteins as a Substrate of Amyloid Fibrils

Several apolipoproteins can be deposited as biochemically distinct forms of amyloid. A rare form of autosomal dominant systemic amyloidosis, involving liver, kidney, skin, cardiac muscle, and respiratory tract, can be caused by deposition of the mutant form of apo AI (Booth *et al.*, 1996; Caballeria *et al.*, 2001; Andreola *et al.*, 2003; Hawkins, 2003). Amyloid fibrils derived from the wt of apo AI can also be found in cholesterol plaques in arteries of elderly humans (Westermarck *et al.*, 1995) and in aged dogs (Johnson *et al.*, 1992; Roertgen *et al.*, 1995). In the majority of cases, the main constituent of fibrils is a 80–93-amino acid long N-terminal fragment of the apo AI protein (Andreola *et al.*, 2003). C-terminal-derived fragments of apo AI can also form fibrils, but this occurs relatively rarely (de Sousa *et al.*, 2000). Apo II (Zeidler *et al.*, 1997; Benson *et al.*, 2001) and IV (Bergstrom *et al.*, 2001) can form amyloid fibrils in humans. One reason for the self-aggregation tendency of apolipoproteins is that apolipoproteins, especially AI, are capable of extensive conformational changes during the transition between free and the lipid-bound forms (Leroy and Jonas, 1994). This allows for specific interactions with apolipoprotein receptor while delivering lipids to the cell (Trigatti *et al.*, 2003). Unfortunately, a subtle increase in the β -sheet content during this conformation transition can lead to disease-associated self-aggregation. Most of the kindreds presenting with apo AI amyloidosis carry a point mutation within its N-terminus, which is thought to be responsible for maintaining a stable structure while the lipoprotein remains in a lipid-free state (Rogers *et al.*, 1998). Studies have shown that peptides homologous to apo C fragments may also form fibrils *in vitro* but this information has limited *in vivo* application (Hatters *et al.*, 2001).

Apo A1 fragments have also been detected in amyloid deposits in AD (Wisniewski and Frangione, 1992; Wisniewski *et al.*, 1995a). More frequently, the C-terminal fragment of apo E containing residues 216–299 codeposit with A β in senile plaques in AD (Wisniewski *et al.*, 1995b). Amphipathic α -helices of the apo E C-terminal fragment, which normally serve as a lipid binding domain, have a natural predisposition to form hydrophobic interactions. Furthermore, it has been demonstrated that peptides homologous to the C-terminal fragment of apo E can aggregate into amyloid fibrils in solution (Wisniewski *et al.*, 1995b), and that they can induce neurofibrillary tangle-like intracellular inclusions in neurons (Huang *et al.*, 2001). These observations point out that although the major constituent of amyloid in AD is A β , other amyloid-associated proteins may also be induced to fold into the same abnormal conformation. This conformational mimicry may initiate and/or further augment fibrillogenesis in AD (Wisniewski *et al.*, 1995a).

8. Apolipoproteins as a Therapeutic Target in Amyloidoses

Understanding the molecular mechanism of amyloid fibril formation and deposition in various amyloidoses may lead to the development of new therapeutic concepts. Targeting amyloid associated proteins that chaperone fibril aggregation has been demonstrated to be a successful method for reducing amyloid load. Pepys *et al.* (2002) have developed a ligand that binds and chelates the serum amyloid P component. The serum amyloid P component, along with apo E, binds to amyloid fibrils *in vivo* in systemic amyloidosis and inhibits the normal degradative process (Gillmore *et al.*, 1997).

Both disease model animals and affected humans treated with this compound in phase I/II clinical trials demonstrate reduced amyloid-A deposition (Pepys *et al.*, 2002). Similarly, molecules capable of inhibiting the interaction between glycosaminoglycans and amyloid fibrils are being developed as a treatment for systemic amyloidosis A and CAA (Kisilevsky *et al.*, 1995). Similar to compounds targeting the serum amyloid P component, the glycosaminoglycan mimetics are being tested in clinical trials (Garceau *et al.*, 2001).

Apo E is another target for pharmacological intervention. Because tg mice overexpressing the mutated form of APP but which have an apo E KO background show significantly reduced amyloid load (Bales *et al.*, 1997), pharmacological inhibition of apo E/A β interaction may mitigate against A β fibril formation and deposition. Ma *et al.* have demonstrated that a synthetic peptide A β 12–28 (the apo E binding sequence on full-length A β) (Strittmatter *et al.*, 1993a; Golabek *et al.*, 1996) can be used as a competitive inhibitor of the binding of full-length A β to apo E. Blocking the binding between apo E and A β results in a significant reduction of fibril formation and increases survival of cultured neurons (Ma *et al.*, 1996). Use of A β 12–28 *in vivo* is limited due to its toxicity and fibrillogenic potential (Gorevic *et al.*, 1987), but a substitution of the valine at residue 18 for proline renders the modified peptide (A β 12–28P) nonfibrillogenic and nontoxic. Unlike the native sequence, A β 12–28P cannot seed new plaques or codeposit on existing lesions. A β 12–28P shows BBB permeability comparable to that of A β 1–40 and its end-protected version, with D-amino acids has a serum half-life of 62.2 ± 18 min. One-month treatment of APP^{K670N/M671L}/PS1^{M146L} double tg AD mice with A β 12–28P resulted in a 63.3% reduction in A β load in the cortex ($p = 0.0047$) and a 61.9% ($p = 0.0048$) reduction in the hippocampus compared to age-matched tg mice that received placebo (Sadowski *et al.*, 2003; Sadowski *et al.*, 2004). No antibodies against A β were detected in the sera of A β 12–28P-treated mice; therefore, the observed therapeutic effect of this peptide could not be attributed to an antibody clearance response. In addition, no toxicity was observed in treated animals (Sadowski *et al.*, 2003, 2004).

9. Abbreviations

A β	β -amyloid
AD	Alzheimer's disease
Act	α 1-antichymotrypsin
Apo A	apolipoprotein A
Apo E	apolipoprotein E
Apo J	apolipoprotein J
APP	amyloid precursor protein
BBB	blood–brain barrier
CAA	cerebral amyloid angiopathy
CJD	Creutzfeldt-Jakob disease
CNS	central nervous system
CSF	cerebrospinal fluid
HDL	high density lipoproteins
IAPP	islet amyloid polypeptide
KO	knock out (in this chapter refers exclusively to gene knock out)
LDL	low-density lipoproteins
LRP	low-density lipoproteins-related protein
vCJD	variant Creutzfeldt-Jakob disease
PNS	peripheral nervous system
PrP	prion protein existing in two conformers PrP ^C C-cellular; PrP ^{Sc} Sc-scrapie

PS	presenilin
RAGE	receptor for advanced glycation end products
sA β	soluble β -amyloid
sCJD	sporadic Creutzfeldt-Jakob disease
SR-A	receptor type A
VLDL	very low-density lipoproteins
wt	wild type

References

- Alonzo, N.C., Hyman, B.T., Rebeck, G.W., and Greenberg, S.M. (1998). Progression of cerebral amyloid angiopathy: accumulation of amyloid-beta 40 in affected vessels. *J. Neuropathol. Exp. Neurol.* 57:353–359.
- Amouyel, P., Vidal, O., Launay, J.M., and Laplanche, J.L. (1994). The apolipoprotein-e alleles as major susceptibility factors for Creutzfeldt-Jakob disease. *Lancet* 344:1315–1318.
- Andreola, A., Bellotti, V., Giorgetti, S., Mangione, P., Obici, L., Stoppini, M., Torres, J., Monzani, E., Merlini, G., and Sunde, M. (2003). Conformational switching and fibrillogenesis in the amyloidogenic fragment of apolipoprotein A-I. *J. Biol. Chem.* 278:2444–2451.
- Antzutkin, O.N., Balbach, J.J., and Tycko, R. (2003). Site-specific identification of non-beta-strand conformations in Alzheimer's beta-amyloid fibrils by solid-state NMR. *Biophys. J.* 84:3326–3335.
- Aucouturier, P., Kacsak, R.J., Frangione, B., and Wisniewski, T. (1999). Biochemical and conformational variability of human prion strains in sporadic Creutzfeldt-Jakob disease. *Neurosci. Lett.* 274:33–36.
- Bales, K.R., Verina, T., Dodel, R.C., Du, Y.S., Altstiel, L., Bender, M., Hyslop, P., Johnstone, E.M., Little, S.P., Cummins, D.J., Piccardo, P., Ghetti, B., and Paul, S.M. (1997). Lack of apolipoprotein E dramatically reduces amyloid β -peptide deposition. *Nat. Genet.* 17:263–264.
- Bales, K.R., Verina, T., Cummins, D.J., Du, Y., Dodel, R.C., Saura, J., Fishman, C.E., DeLong, C.A., Piccardo, P., Petegnief, V., Ghetti, B., and Paul, S.M. (1999). Apolipoprotein E is essential for amyloid deposition in the APPV717F transgenic mouse model of Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* 96:15233–15238.
- Barrow, C.J., Yasuda, A., Kenny, P.T., and Zagorski, M.G. (1992). Solution conformations and aggregational properties of synthetic amyloid beta-peptides of Alzheimer's disease. Analysis of circular dichroism spectra. *J. Mol. Biol.* 225:1075–1093.
- Baumann, M.H., Kallijarvi, J., Lankinen, H., Soto, C., and Haltia, M. (2000). Apolipoprotein E includes a binding site which is recognized by several amyloidogenic polypeptides. *Biochem. J.* 349:77–84.
- Beissinger, M., and Buchner, J. (1998). How chaperones fold proteins. *Biol. Chem.* 379:245–259.
- Benson, M.D., Liepnieks, J.J., Yazaki, M., Yamashita, T., Asl, K.H., Guenther, B., and Kluge-Beckerman, B. (2001). A new human hereditary amyloidosis: the result of a stop-codon mutation in the apolipoprotein AII gene. *Genomics* 72:272–277.
- Bergstrom, J., Murphy, C., Eulitz, M., Weiss, D.T., Westermark, G.T., Solomon, A., and Westermark, P. (2001). Codeposition of apolipoprotein A-IV and transthyretin in senile systemic (ATTR) amyloidosis. *Biochem. Biophys. Res. Commun.* 285:903–908.
- Booth, D.R., Tan, S.Y., Booth, S.E., Tennent, G.A., Hutchinson, W.L., Hsuan, J.J., Totty, N.F., Truong, O., Soutar, A.K., Hawkins, P.N., Bruguera, M., Caballeria, J., Solé, M., Campistol, J.M., and Pepys, M.B. (1996). Hereditary hepatic and systemic amyloidosis caused by a new deletion/insertion mutation in the apolipoprotein A1 gene. *J. Clin. Invest.* 97:2714–2721.
- Bornebroek, M., Haan, J., van Duinen, S.G., Maat Schieman, M.L.C., Van Buchem, M.A., Bakker, E., Van Broeckhoven, C., and Roos, R.A.C. (1997). Dutch hereditary cerebral amyloid angiopathy: structural lesions and apolipoprotein E genotype. *Ann. Neurol.* 41:695–698.
- Boyett, K.W., DiCarlo, G., Jantzen, P.T., Jackson, J., O'Leary, C., Wilcock, D., Morgan, D., and Gordon, M.N. (2003). Increased fibrillar beta-amyloid in response to human C1q injections into hippocampus and cortex of APP + PS1 transgenic mice. *Neurochem. Res.* 28:83–93.
- Boyles, J.K., Zoellner, C.D., Anderson, L.J., Kosik, L.M., Pitas, R.E., Weisgraber, K.H., Hui, D.Y., Mahley, R.W., Gebicke-Haerter, P.J., Ignatias, M.J., and Shooter, E.M. (1989). A role for apolipoprotein E, apoprotein A-I, low density

- lipoprotein receptors in cholesterol transport during regeneration and remyelination of the sciatic nerve. *J. Clin. Invest.* 83:1015–1031.
- Burdick, D., Soreghan, B., Kwon, M., Kosmoski, J., Knauer, M., Henschen, A., Yates, J., Cotman, C., and Glabe, C. (1992). Assembly and aggregation properties of synthetic Alzheimer's A4/beta amyloid peptide analogs. *J. Biol. Chem.* 267:546–554.
- Caballeria, J., Bruguera, M., Sole, M., Campistol, J.M., and Rodes, J. (2001). Hepatic familial amyloidosis caused by a new mutation in the apolipoprotein AI gene: clinical and pathological features. *Am. J. Gastroenterol.* 96: 1872–1876.
- Castano, E.M., Prelli, F., Pras, M., and Frangione, B. (1995a). Apolipoprotein-E carboxyl-terminal fragments are complexed to amyloid-a and amyloid-l-implications for amyloidogenesis and Alzheimers disease. *J. Biol. Chem.* 270: 17610–17615.
- Castano, E.M., Prelli, F., Wisniewski, T., Golabek, A., Kumar, R.A., Soto, C., and Frangione, B. (1995b). Fibrillogenesis in Alzheimers-disease of amyloid-beta peptides and apolipoprotein-e. *Biochem. J.* 306:599–604.
- Castano, E.M., Prelli, F., Wisniewski, T., Golabek, A.A., Kumar, R.A., Soto, C., and Frangione, B. (1995). Fibrillogenesis in Alzheimer's disease of amyloid beta peptides and apolipoprotein E. *Biochem. J.* 306:599–604.
- Chang, Y.T., Tsai, S.F., Wang, W.J., Hong, C.J., Huang, C.Y., and Wong, C.K. (2001). A study of apolipoproteins E and A-I in cutaneous amyloids. *Br. J. Dermatol.* 145:422–427.
- Chapman, J., Cervenakova, L., Lee, H.S., Estupinan, J., Richardson, S., Vnencak-Jones, C.L., Gajdusek, D.C., Korczyn, A.D., Brown, P., and Goldfarb, L.G. (1998). APOE in non-Alzheimer amyloidoses—transmissible spongiform encephalopathies. *Neurology* 51:548–553.
- Choi, N.H., Nakano, Y., Tobe, T., Mazda, T., and Tomita, M. (1990a). Incorporation of Sp-40,40 into the soluble membrane attack complex (Smac, Sc5B-9) of complement. *Int. Immunol.* 2:413–417.
- Choi, N.H., Tobe, T., Hara, K., Yoshida, H., and Tomita, M. (1990b). Sandwich ELISA assay for quantitative measurement of SP-40,40 in seminal plasma and serum. *J. Immunol. Methods* 131:159–163.
- Choi, N.H., Tobe, T., Hara, K., Yoshida, H., and Tomita, M. (1990c). Sandwich Elisa assay for quantitative measurement of Sp-40,40 in seminal plasma and serum. *J. Immunol. Methods* 131:159–163.
- Choi-Miura, N.H., Ihara, Y., Fukuchi, K., Takeda, M., Nakano, Y., Tobe, T., and Tomita, M. (1992). SP-40,40 is a constituent of Alzheimer's amyloid. *Acta Neuropathol.* 83:260–264.
- Corder, E.H., Saunders, A.M., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Small, G.W., Roses, A.D., Haines, J.L., and Pericak-Vance, M.A. (1993). Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* 261:921–923.
- Coria, F., Castano, E.M., Prelli, F., Larrondo-Lillo, M., vanDuinen, S., Shelanski, M.L., and Frangione, B. (1988). Isolation and characterization of amyloid P component from Alzheimer's disease and other types of cerebral amyloidosis. *Lab. Invest.* 58:454–457.
- Cui, D., Hoshii, Y., Takahashi, M., Kawano, H., Iwata, T., and Ishihara, T. (1998). An immunohistochemical study of amyloid P component, apolipoprotein E and ubiquitin in human and murine amyloidosis. *Pathol. Int.* 48:362–367.
- Danik, M., Chabot, J.G., Mercier, C., Benabid, A.L., Chauvin, C., Quirion, R., and Suh, M. (1991). Human gliomas and epileptic foci express high-levels of a messenger-RNA related to rat testicular sulfated glycoprotein-2, a purported marker of cell-death. *Proc. Natl. Acad. Sci. USA* 88:8577–8581.
- Deane, R., Yan, S.D., Subramanyam, R.K., Larue, B., Jovanovic, S., Hogg, E., Welch, D., Manness, L., Lin, C., Yu, J., Zhu, H., Ghiso, J., Frangione, B., Stern, A., Schmidt, A.M., Armstrong, D.L., Arnold, B., Liliensiek, B., Nawroth, P., Hofman, F., Kindy, M., Stern, D., and Zlokovic, B. (2003). RAGE mediates amyloid-beta peptide transport across the blood–brain barrier and accumulation in brain. *Nat. Med.* 9:907–913.
- DeArmond, S.J., Kretschmar, H., and Prusiner, S.B. (2002). Prion diseases. In: Graham, D.I. and Lantos, P. (eds.). *Greenfield's neuropathology*. London: Arnold, pp. 273–324.
- Del Bo, R., Comi, G.P., Bresolin, N., Castelli, E., Conti, E., Degiuli, A., Ausenda, C.D., and Scarlato, G. (1997). The apolipoprotein E ϵ 4 allele causes a faster decline of cognitive performances in Down's syndrome subjects. *J. Neurol. Sci.* 145:87–91.
- Del Bo, R., Comi, G., Giorda, R., Crimi, M., Locatelli, F., Martinelli-Boneschi, F., Pozzoli, U., Castelli, E., Bresolin, N., and Scarlato, G. (2003). The 129 codon polymorphism of the prion protein gene influences earlier cognitive performance in Down Syndrome subjects. *J. Neurol.* 250:688–692.
- de Sousa, M.M., Vital, C., Ostler, D., Fernandes, R., Pouget-Abadie, J., Carles, D., and Saraiva, M.J. (2000). Apolipoprotein AI and transthyretin as components of amyloid fibrils in a kindred with apoAI Leu178His amyloidosis. *Am. J. Pathol.* 156:1911–1917.
- De Strooper, B. (2003). Aph-1, Pen-2, and nicastrin with presenilin generate an active gamma-secretase complex. *Neuron* 38:9–12.

- Diedrich, J.F., Minnigan, H., Carp, R.I., Whitaker, J.N., Race, R., Frey, W., II., and Haase, A.T. (1991). Neuropathological changes in scrapie and Alzheimer's disease are associated with increased expression of apolipoprotein E and cathepsin D in astrocytes. *J. Virol.* 65:4759–4768.
- Dietzsch, E., Murphy, B.F., Kirszbaum, L., Walker, I.D., and Garson, O.M. (1992). Regional localization of the gene for clusterin (Sp-40,40 gene symbol Cli) to human-chromosome 8P12–P21. *Cytogenet. Cell Genet.* 61:178–179.
- Dong, L.M., Wilson, C., Wardell, M.R., Simmons, T., Mahley, R.W., Weisgraber, K.H., and Agard, D.A. (1994). Human apolipoprotein E. Role of arginine 61 in mediating the lipoprotein preferences of the E3 and E4 isoforms. *J. Biol. Chem.* 269:22358–22365.
- Ellis, R.J., Olichney, J.M., Thal, L.J., Mirra, S.S., Morris, J.C., Beekly, D., and Heyman, A. (1996). Cerebral amyloid angiopathy in the brains of patients with Alzheimer's disease: the CERAD experience. *Neurology* 46:1592–1596.
- Fadika, G.O., and Baumann, M. (2002). Peptides corresponding to gelsolin derived amyloid of the Finnish type (AGel(FIN)) adopt two distinct forms in solution of which only one can polymerize into amyloid fibrils and form complexes with apoE. *Amyloid J. Protein Fold. Disord.* 9:75–82.
- Frangione, B., Wisniewski, T., and Ghiso, J. (1994). Chaperoning Alzheimer's amyloid. *Neurobiol. Aging* 15:97–99.
- Fukumoto, H., Cheung, B.S., Hyman, B.T., and Irizarry, M.C. (2002). Beta-secretase protein and activity are increased in the neocortex in Alzheimer disease. *Arch. Neurol.* 59:1381–1389.
- Fukumoto, H., Rosene, D.L., Moss, M.B., Raju, S., Hyman, B.T., and Irizarry, M.C. (2004). Beta-secretase activity increases with aging in human, monkey, and mouse brain. *Am. J. Pathol.* 164:719–725.
- Furumoto, H., Shimizu, T., Muto, M., Hashimoto, Y., and Nakamura, K. (2002). Apolipoprotein E4 is associated with primary localized cutaneous amyloidosis. *J. Invest. Dermatol.* 119:532–533.
- Gallo, G., Wisniewski, T., Choi-Miura, N.H., Ghiso, J., and Frangione, B. (1994). Potential role of apolipoprotein-E in fibrillogenesis. *Am. J. Pathol.* 145:526–530.
- Gandy, S., Caporaso, G., Buxbaum, J., Frangione, B., and Greengard, P. (1994). APP processing, A β -amyloidogenesis, and the pathogenesis of Alzheimer's disease. *Neurobiol. Aging* 15:253–256.
- Garceau, D., Gurbindo, C., and Laurin, J. (2001). Safety, tolerability and pharmacokinetic profile of Fibrillex™ (anti-AA amyloid agent) in healthy and renal impaired subjects. In: Bely, M. and Apathy, A. (eds). *Proc. IXth Int. Symp. Amyloidosis*, Budapest, pp. 116–118.
- Gejyo, F., Suzuki, S., Kimura, H., Imura, T., Ei, I., Hasegawa, H., and Arakawa, M. (1997). Increased risk of dialysis-related amyloidosis in patients with the apolipoprotein E4 allele. *Amyloid Int. J. Exp. Clin. Invest.* 4:13–17.
- Gherzi-Egea, J.F., Gorevic, P.D., Ghiso, J., Frangione, B., Patlak, C.S., and Fenstermacher, J.D. (1996). Fate of cerebrospinal fluid-borne amyloid β -peptide: rapid clearance into blood and appreciable accumulation by cerebral arteries. *J. Neurochem.* 67:880–883.
- Ghiso, J., Matsubara, E., Koudinov, A., Choi-Miura, N.H., Tomita, M., Wisniewski, T., and Frangione, B. (1993). The cerebrospinal-fluid soluble form of Alzheimer's amyloid beta is complexed to SP-40,40 (apolipoprotein J), an inhibitor of the complement membrane-attack complex. *Biochem. J.* 293:27–30.
- Ghiso, J., Wisniewski, T., and Frangione, B. (1994). Unifying features of systemic and cerebral amyloidosis. *Mol. Neurobiol.* 8:49–64.
- Ghiso, J., Calero, M., Matsubara, E., Governale, S., Chuba, J., Beavis, R., and Wisniewski, T. (1997). Alzheimer's soluble amyloid β is a normal component of urine. *FEBS Lett.* 408:105–108.
- Ghiso, J., Vidal, R., Rostagno, A., Miravalle, L., Holton, J.L., Mead, S., Revesz, T., Plant, G., and Frangione, B. (2000). Amyloidogenesis in familial British dementia is associated with a genetic defect on chromosome 13. *Mol. Basis Dementia* 920:84–92.
- Gillmore, J.D., Hawkins, P.N., and Pepys, M.B. (1997). Amyloidosis: a review of recent diagnostic and therapeutic developments. *Br. J. Haematol.* 99:245–256.
- Golabek, A.A., Marques, M., Lalowski, M., and Wisniewski, T. (1995). Amyloid β binding proteins in vitro and in normal human cerebrospinal fluid. *Neurosci. Lett.* 191:79–82.
- Golabek, A.A., Soto, C., Vogel, T., and Wisniewski, T. (1996). The interaction between apolipoprotein E and Alzheimer's amyloid β -peptide is dependent on β -peptide conformation. *J. Biol. Chem.* 271:10602–10606.
- Golabek, A.A., Kida, E., Walus, M., Perez, C., Wisniewski, T., and Soto, C. (2000). Sodium dodecyl sulfate-resistant complexes of Alzheimer's amyloid β -peptide with the N-terminal, receptor binding domain of apolipoprotein E. *Biophys. J.* 79:1008–1015.
- Gorevic, P.D., Castaño, E.M., Sarma, R., and Frangione, B. (1987). Ten to fourteen residue peptides of Alzheimer's disease protein are sufficient for amyloid fibril formation and its characteristic x-ray diffraction pattern. *Biochem. Biophys. Res. Commun.* 147:854–862.

- Gouras, G.K., Tsai, J., Naslund, J., Vincent, B., Edgar, M., Checler, F., Greenfield, J.P., Haroutunian, V., Buxbaum, J.D., Xu, H.X., Greengard, P., and Relkin, N.R. (2000a). Intraneuronal A beta 42 accumulation in human brain. *Am. J. Pathol.* 156:15–20.
- Gouras, G.K., Xu, H.X., and Edgar, M. (2000b). Intraneuronal beta-amyloid with aging and Alzheimer's disease. *J. Neuropathol. Exp. Neurol.* 59:421.
- Greenberg, S.M., Briggs, M.E., Hyman, B.T., Kokoris, G.J., Takis, C., Kanter, D.S., Kase, C.S., and Pessin, M.S. (1996). Apolipoprotein E ϵ 4 is associated with the presence and earlier onset of hemorrhage in cerebral amyloid angiopathy. *Stroke* 27:1333–1337.
- Greenberg, S.M., Vonsattel, J.P.G., Segal, A.Z., Chiu, R.I., Clatworthy, A.E., Liao, A., Hyman, B.T., and Rebeck, G.W. (1998). Association of apolipoprotein E epsilon 2 and vasculopathy in cerebral amyloid angiopathy. *Neurology* 50: 961–965.
- Griswold, M.D., Roberts, K., and Bishop, P. (1986). Purification and characterization of a sulfated glycoprotein secreted by sertoli cells. *Biochemistry* 25:7265–7270.
- Haltia, M., Prelli, F., Ghiso, J., Kiuru, S., Somer, H., Palo, J., and Frangione, B. (1990). Amyloid protein in familial amyloidosis (Finnish type) is homologous to gelsolin, an actin-binding protein. *Biochem. Biophys. Res. Commun.* 167:927–932.
- Hartmann, K., Rauch, J., Urban, J., Parczyk, K., Diel, P., Pilarsky, C., Appel, D., Haase, W., Mann, K., Weller, A., and Kochbrandt, C. (1991). Molecular-cloning of Gp 80, a glycoprotein complex secreted by kidney-cells in vitro and in vivo—a link to the reproductive-system and to the complement cascade. *J. Biol. Chem.* 266: 9924–9931.
- Hasegawa, H., Nishi, S.I., Ito, S., Saeki, T., Kuroda, T., Kimura, H., Watababe, T., Nakano, M., Gejyo, F., and Arakawa, M. (1996). High prevalence of serum apolipoprotein E4 isoprotein in rheumatoid arthritis patients with amyloidosis. *Arthritis Rheum.* 39:1728–1732.
- Hatters, D.M., Lindner, R.A., Carver, J.A., and Howlett, G.J. (2001). The molecular chaperone, alpha-crystallin, inhibits amyloid formation by apolipoprotein C-II. *J. Biol. Chem.* 276:33755–33761.
- Hawkins, P.N. (2003). Hereditary systemic amyloidosis with renal involvement. *J. Nephrol.* 16:443–448.
- Hilbich, C., Kisters-Woike, B., Reed, J., Masters, C.L., and Beyreuther, K. (1991). Aggregation and secondary structure of synthetic amyloid beta A4 peptides of Alzheimer's disease. *J. Mol. Biol.* 218:149–163.
- Holtzman, D.M., Bales, K.R., Wu, S., Bhat, P., Parsadanian, M., Fagan, A.M., Chang, L.K., Sun, Y., and Paul, S.M. (1999). Expression of human apolipoprotein E reduces amyloid-beta deposition in a mouse model of Alzheimer's disease. *J. Clin. Invest.* 103:R15–R21.
- Holtzman, D.M., Bales, K.R., Tenkova, T., Fagan, A.M., Parsadanian, M., Sartorius, L.J., Mackey, B., Olney, J., McKeel, D., Wozniak, D., and Paul, S.M. (2000a). Apolipoprotein E isoform-dependent amyloid deposition and neuritic degeneration in a mouse model of Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* 97:2892–2897.
- Holtzman, D.M., Fagan, A.M., Mackey, B., Tenkova, T., Sartorius, L., Paul, S.M., Bales, K.R., Hsiao Ashe, K., Irizarry, M.C., and Hyman, B.T. (2000b). Apolipoprotein E facilitates neuritic and cerebrovascular plaque formation in an Alzheimer's disease model. *Ann. Neurol.* 47:739–747.
- Huang, Y.D., Liu, X.Q., Wyss-Coray, T., Brecht, W.J., Sanan, D.A., and Mahley, R.W. (2001). Apolipoprotein E fragments present in Alzheimer's disease brains induce neurofibrillary tangle-like intracellular inclusions in neurons. *Proc. Natl. Acad. Sci. USA* 98:8838–8843.
- Ida, N., Masters, C.L., and Beyreuther, K. (1996). Rapid cellular uptake of Alzheimer amyloid β A4 peptide by cultured human neuroblastoma cells. *FEBS Lett.* 394:174–178.
- Ingelsson, M., Fukumoto, H., Newell, K.L., Growdon, J.H., Hedley-Whyte, E.T., Frosch, M.P., Albert, M.S., Hyman, B.T., and Irizarry, M.C. (2004). Early A beta accumulation and progressive synaptic loss, gliosis, and tangle formation in AD brain. *Neurology* 62:925–931.
- Ji, Y., Permanne, B., Sigurdsson, E.M., Holtzman, D.M., and Wisniewski, T. (2001). Amyloid β 40/42 clearance across the blood–brain barrier following intra-ventricular injections in wild-type, apoE knock-out and human apoE3 or E4 expressing transgenic mice. *J. Alzheimer's Dis.* 3:23–30.
- Ji, Y., Gong, Y., Gan, W., Beach, T., Holtzman, D.M., and Wisniewski, T. (2003). Apolipoprotein E isoform-specific regulation of dendritic spine morphology in apolipoprotein E transgenic mice and Alzheimer's disease patients. *Neuroscience* 122:305–315.
- Johnson, K.H., Sletten, K., Hayden, D.W., O'Brien, T.D., Roertgen, K.E., and Westermark, P. (1992). Pulmonary vascular amyloidosis in aged dogs—a new form of spontaneously occurring amyloidosis derived from apolipoprotein Ai. *Am. J. Pathol.* 141:1013–1019.

- Johnson, L.V., Leitner, W.P., Rivest, A.J., Staples, M.K., Radeke, M.J., and Anderson, D.H. (2002). The Alzheimer's A beta-peptide is deposited at sites of complement activation in pathologic deposits associated with aging and age-related macular degeneration. *Proc. Natl. Acad. Sci. USA* 99:11830–11835.
- Kalaria, R.N., and Premkumar, D.R.D. (1995). Apolipoprotein E genotype and cerebral amyloid angiopathy. *Lancet* 346:1424.
- Kalaria, R.N., Cohen, D.L., and Premkumar, D.R.D. (1996). Apolipoprotein E alleles and brain vascular pathology in Alzheimer's disease. *Ann. N. Y. Acad. Sci.* 777:266–270.
- Kindy, M.S., and Rader, D.J. (1998). Reduction in amyloid A amyloid formation in apolipoprotein-E-deficient mice. *Am. J. Pathol.* 152:1387–1395.
- Kirschner, D.A., Inouye, H., Duffy, L.K., Sinclair, A., Linda, M., and Selkoe, D.J. (1987). Synthetic peptide homologous to β protein from Alzheimer's disease forms amyloid-like fibrils in vitro. *Proc. Natl. Acad. Sci. USA* 84: 6953–6957.
- Kirsbaum, L., Bozas, S.E., and Walker, I.D. (1992). Sp-40,40, a protein involved in the control of the complement pathway, possesses a unique array of disulfide bridges. *FEBS Lett.* 297:70–76.
- Kisilevsky, R., Lemieux, L.J., Fraser, P.E., Kong, X., Hultin, P.G., and Szarek, W.A. (1995). Arresting amyloidosis in vivo using small-molecule anionic sulphonates or sulphates: implications for Alzheimer's disease. *Nat. Med.* 1: 143–148.
- Knaus, K.J., Morillas, M., Swietnicki, W., Malone, M., Surewicz, W.K., and Yee, V.C. (2001). Crystal structure of the human prion protein reveals a mechanism for oligomerization. *Nat. Struct. Biol.* 8:770–774.
- Kostner, G.M. (1989). Lipoprotein receptors and atherosclerosis. *Biochem. Soc. Trans.* 17:639–641.
- Koudinov, A., Matsubara, E., Frangione, B., and Ghiso, J. (1994). The soluble form of Alzheimer's amyloid beta protein is complexed to high density lipoprotein 3 and very high density lipoprotein in normal human plasma. *Biochem. Biophys. Res. Commun.* 205:1164–1171.
- LaFerla, F.M., Troncoso, J.C., Strickland, D.K., Kawas, C.H., and Jay, G. (1997). Neuronal cell death in Alzheimer's disease correlates with apoE uptake and intracellular A β stabilization. *J. Clin. Invest.* 100:310–320.
- Leroy, A., and Jonas, A. (1994). Native-like structure and self-association behavior of apolipoprotein-A-I in a water N-propanol solution. *Biochim. Biophys. Acta Lipids Lipid Metab.* 1212:285–294.
- Levy, E., Lopez-Otin, C., Ghiso, J., and Geltner, D. (1989). Stroke in Icelandic patients with hereditary amyloid angiopathy is related to a mutation in the cystatin C gene, and inhibitor of cysteine proteases. *J. Exp. Med.* 169: 1771–1778.
- Levy, E., Carman, M.D., Fernandez-Madrid, I., Lieberburg, I., Power, M.D., vanDuinen, S.G., Bots, G.Th.A.M., Luyendijk, W., and Frangione, B. (1990a). Mutation of the Alzheimer's disease amyloid gene in hereditary cerebral hemorrhage, Dutch type. *Science* 248:1124–1126.
- Levy, E., Haltia, M., Fernandez-Madrid, I., and Frangione, B. (1990b). Mutation in gelsolin gene in Finnish hereditary amyloidosis. *J. Exp. Med.* 172:1865–1867.
- Lewis, J., Dickson, D., Lin, W.L., Chisholm, L., Corral, A., Jones, G., Yen, S.H., Sahara, N., Skipper, L., Yager, D., Eckman, C., Hardy, J., Hutton, M., and McGowan, E. (2001). Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. *Nature* 293:1489–1491.
- Ma, J., Yee, A., Brewer, H.B., Jr., Das, S., and Potter, H. (1994). Amyloid-associated proteins alpha 1-antichymotrypsin and apolipoprotein E promote assembly of Alzheimer beta-protein into filaments. *Nature* 372:92–94.
- Ma, J., Brewer, B.H., Potter, H., and Brewer, H.B., Jr. (1996). Alzheimer A β neurotoxicity: promotion by antichymotrypsin, apoE4; inhibition by A β -related peptides. *Neurobiol. Aging* 17:773–780.
- Mackic, J.B., McComb, J.G., Calero, M., Ghiso, J., Kim, K.S., Yan, S.D., Stern, D., Schmidt, A.M., Frangione, B., and Zlokovic, B.V. (1998). Human blood-brain barrier receptors for Alzheimer's amyloid-beta 1–40. Asymmetrical binding, endocytosis, and transcytosis at the apical side of brain microvascular endothelial cell monolayer. *J. Clin. Invest.* 102:734–743.
- Mackic, J.B., Bading, J., Ghiso, J., Walker, L., Wisniewski, T., Frangione, B., and Zlokovic, B. (2002). Transport across the blood-brain barrier and differential cerebrovascular sequestration of circulating Alzheimer's amyloid- β peptide in aged Rhesus versus aged Squirrel monkeys. *Vasc. Pharmacol.* 38:303–313.
- Maestre, G., Otman, R., Stern, Y., Gurland, B., Chun, M., Tang, M.X., Shelanski, M., Tycko, B., and Mayeux, R. (1995). Apolipoprotein E and Alzheimer's disease: ethnic variation in genotypic risks. *Ann. Neurol.* 37:254–259.
- Mahley, R.W. (1988). Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* 240:622–630.
- Mahley, R.W., Nathan, B.P., and Pitas, R.E. (1996). Apolipoprotein E—structure, function, and possible roles in Alzheimer's disease. *Ann. N. Y. Acad. Sci.* 777:139–145.

- Mann, D.M.A., Pickering-Brown, S.M., Takeuchi, A., and Iwatsubo, T. (2001). Amyloid angiopathy and variability in amyloid beta deposition is determined by mutation position in presenilin-1-linked Alzheimer's disease. *Am. J. Pathol.* 158:2165–2175.
- Masliah, E., Samuel, W., Veinbergs, I., Mallory, M., Mante, M., and Saitoh, T. (1997). Neurodegeneration and cognitive impairment in apoE-deficient mice is ameliorated by infusion of recombinant apoE. *Brain Res.* 751:307–314.
- Matsubara, E., Frangione, B., and Ghiso, J. (1995). Characterization of apolipoprotein J–Alzheimer's A beta interaction. *J. Biol. Chem.* 270:7563–7567.
- Mayeux, R., Stern, Y., Ottman, R., Tatemichi, T.K., Tang, M.X., Maestre, G., Ngai, C., Tycko, B., and Ginsberg, H. (1993). The apolipoprotein epsilon 4 allele in patients with Alzheimer's disease. *Ann. Neurol.* 34:752–754.
- Mazur-Kolecka, B., Frackowiak, J., and Wisniewski, H.M. (1995). Apolipoproteins E3 and E4 induce, and transthyretin prevents accumulation of the Alzheimer's β -amyloid peptide in cultured vascular smooth muscle cells. *Brain Res.* 698:217–222.
- Merlini, G., and Bellotti, V. (2003). Molecular mechanisms of amyloidosis. *N. Engl. J. Med.* 349:583–596.
- Merz, P.A., Wisniewski, H.M., Somerville, R.A., Bobin, S.A., Masters, C.L., and Iqbal, K. (1983). Ultrastructural morphology of amyloid fibrils from neuritic and amyloid plaques. *Acta Neuropathol.* 60:113–124.
- Monro, O.R., Mackic, J.B., Yamada, S., Segal, M.B., Ghiso, J., Maurer, C., Calero, M., Frangione, B., and Zlokovic, B.V. (2002). Substitution at codon 22 reduces clearance of Alzheimer's amyloid-beta peptide from the cerebrospinal fluid and prevents its transport from the central nervous system into blood. *Neurobiol. Aging* 23:405–412.
- Mori, C., Spooner, E.T., Wisniewski, K.E., Wisniewski, T.M., Yamaguchi, H., Saido, T.C., Li, C., Tolan, D.R., Selkoe, D., and Lemere, C.A. (2002). Intraneuronal A β 42 accumulation in Down syndrome brain. *Amyloid* 9:88–102.
- Mufson, E.J., Benzing, W.C., Cole, G.M., Wang, H., Emerich, D.F., Sladek, J.R., Morrison, J.H., and Kordower, J.H. (1994). Apolipoprotein E-immunoreactivity in aged rhesus-monkey cortex—colocalization with amyloid plaques. *Neurobiol. Aging* 15:621–627.
- Nakagawa, Y., Kitamoto, T., Furukawa, H., Ogomori, K., and Tateishi, J. (1995). Apolipoprotein-e in Creutzfeldt-Jakob Disease. *Lancet* 345:68.
- Namba, Y., Tomonaga, M., Kawasaki, H., Otomo, E., and Ikeda, K. (1991). Apolipoprotein E immunoreactivity in cerebral amyloid deposits and neurofibrillary tangles in Alzheimer's disease and kuru plaque amyloid in Creutzfeldt-Jakob disease. *Brain Res.* 41:163–166.
- Namba, Y., Tsuchiya, H., and Ikeda, K. (1992). Apolipoprotein B immunoreactivity in senile plaque and vascular amyloids and neurofibrillary tangles in the brains of patients with Alzheimer's disease. *Neurosci. Lett.* 134:264–266.
- Naslund, J., Thyberg, J., Tjernberg, L.O., Wernstedt, C., Karlstrom, A.R., Bogdanovic, N., Gandy, S.E., Lannfelt, L., Terenius, L., and Nordstedt, C. (1995). Characterization of stable complexes involving apolipoprotein E and the amyloid beta peptide in Alzheimer's disease brain. *Neuron* 15:219–228.
- Nathan, B.P., Bellosa, S., Sanan, D.A., Weisgraber, K.H., Mahley, R.W., and Pitas, R.E. (1994). Differential effects of apolipoproteins E3 and E4 on neuronal growth in vitro. *Science* 264:850–852.
- Nochlin, D., Bird, T.D., Nemens, E.J., Ball, M.J., and Sumi, S.M. (1998). Amyloid angiopathy in a Volga German family with Alzheimer's disease and a presenilin-2 mutation ((NI)-I-141). *Ann. Neurol.* 43:131–135.
- Paresce, D.M., Ghosh, R.N., and Maxfield, F.R. (1996). Microglial cells internalize aggregates of the Alzheimer's disease amyloid beta-protein via a scavenger receptor. *Neuron* 17:553–565.
- Pepys, M.B., Herbert, J., Hutchinson, W.L., Tennent, G.A., Lachmann, H.J., Gallimore, J.R., Lovat, L.B., Bartfai, T., Alanine, A., Hertel, C., Hoffmann, T., Jakob-Roetne, R., Norcross, R.D., Kemp, J.A., Yamamura, K., Suzuki, M., Taylor, G.W., Murray, S., Thompson, D., Purvis, A., Kolstoe, S., Wood, S.P., and Hawkins, P.N. (2002). Targeted pharmacological depletion of serum amyloid P component for treatment of human amyloidosis. *Nature* 417: 254–259.
- Petkova, A.T., Ishii, Y., Balbach, J.J., Antzutkin, O.N., Leapman, R.D., Delaglio, F., and Tycko, R. (2002). A structural model for Alzheimer's beta-amyloid fibrils based on experimental constraints from solid state NMR. *Proc. Natl. Acad. Sci. USA* 99:16742–16747.
- Petkova, A.T., Buntkowsky, G., Dyda, F., Leapman, R.D., Yau, W.M., and Tycko, R. (2004). Solid state NMR reveals a pH-dependent antiparallel beta-sheet registry in fibrils formed by a beta-amyloid peptide. *J. Mol. Biol.* 335: 247–260.
- Pillot, T., Goethals, M., Vanloo, B., Lins, L., Brasseur, R., Vandekerckhove, J., and Rosseneu, M. (1997). Specific modulation of the fusogenic properties of the Alzheimer β -amyloid peptide by apolipoprotein E isoforms. *Eur. J. Biochem.* 243:650–659.
- Pitas, R.E., Boyles, J.K., Lee, S.H., Hui, D., and Weisgraber, K.H. (1987). Lipoproteins and their receptors in the central-nervous-system—characterization of the lipoproteins in cerebrospinal-fluid and identification of apolipoprotein-B,E(Ldl) receptors in the brain. *J. Biol. Chem.* 262:14352–14360.

- Poduslo, J.F., Curran, G.L., Sanyal, B., and Selkoe, D.J. (1999). Receptor-mediated transport of human amyloid β -protein 1–40 and 1–42 at the blood–brain barrier. *Neurobiol. Dis.* 6:190–199.
- Potter, H., Wefes, I.M., and Nilsson, L.N. (2001). The inflammation-induced pathological chaperones ACT and apo-E are necessary catalysts of Alzheimer amyloid formation. *Neurobiol. Aging* 22:923–930.
- Premkumar, D.R.D., Cohen, D.L., Hedera, P., Friedland, R.P., and Kalaria, R.N. (1996). Apolipoprotein E- ϵ 4 alleles in cerebral amyloid angiopathy and cerebrovascular pathology associated with Alzheimer's disease. *Am. J. Pathol.* 148:2083–2095.
- Prusiner, S.B., Bolton, D.C., Bowman, K.A., Cochran, S.P., Groth, D.F., and McKinley, M.P. (1983a). Identification of a 30,000 molecular-weight protein purifying with scrapie prions. *Clin. Res.* 31:A498.
- Prusiner, S.B., Bolton, D.C., Bowman, K.A., Cochran, S.P., Groth, D.F., and McKinley, M.P. (1983b). Properties of a 30,000Mw protein-component of the scrapie prion. *Fed. Proc.* 42:849.
- Prusiner, S.B., McKinley, M.P., Bowman, K.A., Bolton, D.C., Bendheim, P.E., Groth, D.F., and Glenner, G.G. (1983c). Scrapie prions aggregate to form amyloid-like birefringent rods. *Cell* 35:349–358.
- Rader, D.J. (2003). Regulation of reverse cholesterol transport and clinical implications. *Am. J. Cardiol.* 92:42J–49J.
- Rebeck, G.W., Reiter, J.S., Strickland, D.K., and Hyman, B.T. (1993). Apolipoprotein E in sporadic Alzheimer's disease: allelic variation and receptor interactions. *Neuron* 11:575–580.
- Relkin, N.R., Tanzi, R., Breitner, J., Farrer, L., Gandy, S., Haines, J., Hyman, B., Mullan, M., Poirer, J., Strittmatter, W., Folstein, M., Farlow, M., Mayeux, R., Petersen, R., Roses, A., Schenk, D., Small, G., Van Gool, W., Cook-Deegan, R., Fleck, L., Kapp, M., Karlinsky, H., Pericak-Vance, M., and Post, S. (1996). Apolipoprotein E genotyping in Alzheimer's disease. *Lancet* 347:1091–1095.
- Revesz, T., Holton, J.L., Lashley, T., Plant, G., Rostagno, A., Ghiso, J., and Frangione, B. (2002). Sporadic and familial cerebral amyloid angiopathies. *Brain Pathol.* 12:343–357.
- Revesz, T., Ghiso, J., Lashley, T., Plant, G., Rostagno, A., Frangione, B., and Holton, J.L. (2003). Cerebral amyloid angiopathies: a pathologic, biochemical, and genetic view. *J. Neuropathol. Exp. Neurol.* 62:885–898.
- Roertgen, K.E., Lund, E.M., O'Brien, T.D., Westermarck, P., Hayden, D.W., and Johnson, K.H. (1995). Apolipoprotein AI-derived pulmonary vascular amyloid in aged dogs. *Am. J. Pathol.* 147:1311–1317.
- Rogers, D.P., Roberts, L.M., Lebowitz, J., Datta, G., Anantharamaiah, G.M., Engler, J.A., and Brouillette, C.G. (1998). The lipid-free structure of apolipoprotein A-I: effects of amino-terminal deletions. *Biochemistry* 37:11714–11725.
- Rostagno, A., Magnotti, L., Tomidokoro, Y., Frangione, B., Ghiso, J., Revesz, T., Lashley, T., and Holton, J. (2002). Complement activation in chromosome 13 dementias. *Neurobiol. Aging* 23:S454–S455.
- Sadowski, M., Ji, Y., Scholtzova, H., Sigurdsson, E.M., and Wisniewski, T. (2003). Blocking apolipoprotein E/ β -amyloid interaction as a therapeutic approach for Alzheimer's disease. *Neurology* 60:68.
- Sadowski, M., Pankiewicz, J., Scholtzova, H., Ripellino, J., Li, Y., Schmidt, S.D., Mathews, P.M., Fryer, J., Holtzman, D., Sigurdsson, E.M., and Wisniewski, T. (2004). A synthetic peptide blocking the apolipoprotein E/ β -amyloid binding mitigates β -amyloid toxicity and fibril formation *in vitro* and reduces β -amyloid plaques in transgenic mice. *Am. J. Pathol.* 165:937–948.
- Salvatore, M., Seeber, A.C., Nacmias, B., Petraroli, R., D'Alessandro, M., Sorbi, S., and Pocchiari, M. (1995). Apolipoprotein E in sporadic and familial Creutzfeldt-Jakob disease. *Neurosci. Lett.* 199:95–98.
- Sanan, D.A., Weisgraber, K.H., Russell, S.J., Mahley, R.W., Huang, D., Saunders, A., Schmechel, D., Wisniewski, T., Frangione, B., Roses, A.D., and Strittmatter, W.J. (1994). Apolipoprotein E associates with β -amyloid peptide of Alzheimer's disease to form novel monofibrils. Isoform apoE4 associates more efficiently than apoE3. *J. Clin. Invest.* 94:860–869.
- Saunders, A.M., Schmechel, K., Breitner, J.C.S., Benson, M.D., Brown, W.T., Goldfarb, L., Goldgaber, D., Manwaring, M.G., Szymanski, M.H., McCown, N., Dole, K.C., Schmechel, D.E., Strittmatter, W.J., Pericakvance, M.A., and Roses, A.D. (1993a). Apolipoprotein E-epsilon-4 allele distributions in late-onset Alzheimer's disease and in other amyloid-forming diseases. *Lancet* 342:710–711.
- Saunders, A.M., Strittmatter, W.J., Schmechel, D., Georgehyslop, P.H.S., Pericakvance, M.A., Joo, S.H., Rosi, B.L., Gusella, J.F., Crappermaclachlan, D.R., Alberts, M.J., Hulette, C., Crain, B., Goldgaber, D., and Roses, A.D. (1993b). Association of apolipoprotein-e allele epsilon-4 with late-onset familial and sporadic Alzheimer's disease. *Neurology* 43:1467–1472.
- Saunders, A.M., Strittmatter, W.J., Schmechel, D., Pericakvance, M.A., and Roses, A.D. (1993c). Apolipoprotein E4 is associated with late-onset familial and sporadic Alzheimer's disease. *Am. J. Hum. Genet.* 53:152.
- Schmechel, D.E., Saunders, A.M., Strittmatter, W.J., Crain, B.J., Hulette, C.M., Joo, S.H., Pericak-Vance, M.A., Goldgaber, D., and Roses, A.D. (1993). Increased amyloid β -peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease. *Proc. Natl. Acad. Sci. USA* 90:9649–9653.
- Schupf, N. (2002). Genetic and host factors for dementia in Down's syndrome. *Br. J. Psychiatry* 180:405–410.

- Schupf, N., Kapell, D., Lee, J.H., Zigman, W., Canto, B., Tycko, B., and Mayeux, R. (1996). Onset of dementia is associated with apolipoprotein E ϵ 4 in Down's syndrome. *Ann. Neurol.* 40:799–801.
- Segrest, J.P., Garber, D.W., Brouillette, C.G., Harvey, S.C., and Anantharamaiah, G.M. (1994). The amphipathic α -helix—a multifunctional structural motif in plasma apolipoproteins. *Adv. Protein Chem.* 45:303–369.
- Selkoe, D.J. (1995). Alzheimer's disease. Missense on the membrane. *Nature* 375:734–735.
- Selkoe, D.J. (1997). Neuroscience—Alzheimer's disease: genotypes, phenotype, and treatments. *Science* 275:260–231.
- Selkoe, D.J. (2000). The origins of Alzheimer disease: A is for amyloid. *J. Am. Med. Assoc.* 283:1615–1617.
- Selkoe, D.J. (2003). Folding proteins in fatal ways. *Nature* 426:900–904.
- Shibata, M., Yamada, S., Kumar, S., Calero, M., Bading, J., Frangione, B., Holtzman, D., Miller, C.A., Strickland, D.K., Ghiso, J., and Zlokovic, B. (2002). Clearance of Alzheimer's amyloid- β 1–40 peptide from brain by LDL receptor-related protein-1 at the blood–brain barrier. *J. Clin. Invest.* 106:1489–1499.
- Shuvaev, V.V. and Siest, G. (1996). Interaction between human amphipathic apolipoproteins and amyloid β -peptide: surface plasmon resonance studies. *FEBS Lett.* 383:9–12.
- Snow, A.D., Willner, J., and Kisilevsky, R. (1987). Sulphated glycosaminoglycans: a common constituent of all amyloid? *Lab. Invest.* 56:120–124.
- Snow, A.D., Kisilevsky, R., Willmer, J., Prusiner, S.B., and DeArmond, S.J. (1989). Sulfated glycosaminoglycans in amyloid plaques of prion diseases. *Acta Neuropathol.* 77:337–342.
- Snow, A.D., Wight, T.N., Nochlin, D., Koike, Y., Kimata, K., DeArmond, S.J., and Prusiner, S.B. (1990). Immunolocalization of heparin sulfate proteoglycans to the prion amyloid plaques of Gerstmann-Straussler-Scheinker syndrome, Creutzfeldt-Jacob disease and scrapie. *Lab. Invest.* 63:601–611.
- Soto, C., Castaño, E.M., Prelli, F., Kumar, R.A., and Baumann, M. (1995). Apolipoprotein E increases the fibrillogenic potential of synthetic peptides derived from Alzheimer's, gelsolin and AA amyloids. *FEBS Lett.* 371:110–114.
- Steiner, H., Revesz, T., Neumann, M., Romig, H., Grim, M.G., Pesold, B., Kretschmar, H.A., Hardy, J., Holton, J.L., Baumeister, R., Houlden, H., and Haass, C. (2001). A pathogenic presenilin-1 deletion causes aberrant A β 42 production in the absence of congophilic amyloid plaques. *J. Biol. Chem.* 276:7233–7239.
- Stine, W.B., Dahlgren, K.N., Krafft, G.A., and LaDu, M.J. (2003). In vitro characterization of conditions for amyloid- β peptide oligomerization and fibrillogenesis. *J. Biol. Chem.* 278:11612–11622.
- Strittmatter, W.J., Saunders, A.M., Schmechel, D., Pericak-Vance, M., Enghild, J., Salvesen, G.S., and Roses, A.D. (1993a). Apolipoprotein E: high-avidity binding to β -amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc. Natl. Acad. Sci. USA* 90:1977–1981.
- Strittmatter, W.J., Weisgraber, K.H., Huang, D.Y., Dong, L.M., Salvesen, G.S., Pericak-Vance, M., Schmechel, D., Saunders, A.M., Goldgaber, D., and Roses, A.D. (1993b). Binding of human apolipoprotein E to synthetic amyloid β peptide: isoform-specific effects and implications for late-onset Alzheimer disease. *Proc. Natl. Acad. Sci. USA* 90:8098–8102.
- Takahashi, M., Hoshii, Y., Kawano, H., Gondo, T., Yokota, T., Okabayashi, H., Shimada, I., and Ishihara, T. (1998). Ultrastructural evidence for the formation of amyloid fibrils within cardiomyocytes in isolated atrial amyloid. *Amyloid Int. J. Exp. Clin. Invest.* 5:35–42.
- Takahashi, R.H., Almeida, C.G., Kearney, P.F., Yu, F.M., Lin, M.T., Milner, T.A., and Gouras, G.K. (2004). Oligomerization of Alzheimer's β -amyloid within processes and synapses of cultured neurons and brain. *J. Neurosci.* 24:3592–3599.
- Takao, M., Ghetti, B., Hayakawa, I., Ikeda, E., Fukuuchi, Y., Miravalle, L., Piccardo, P., Murrell, J.R., Glazier, B.S., and Koto, A. (2002). A novel mutation (G217D) in the Presenilin 1 gene (PSEN1) in a Japanese family: presenile dementia and parkinsonism are associated with cotton wool plaques in the cortex and striatum. *Acta Neuropathol.* 104:155–170.
- Tatzelt, J., Maeda, N., Pekny, M., Yang, S.L., Betsholtz, C., Eliasson, C., Cayetano, J., Camerino, A.P., DeArmond, S.J., and Prusiner, S.B. (1996). Scrapie in mice deficient in apolipoprotein E or glial fibrillary acidic protein. *Neurology* 47:449–453.
- Thal, D.R., Ghebremedhin, E., Rub, U., Yamaguchi, H., Del Tredici, K., and Braak, H. (2002). Two types of sporadic cerebral amyloid angiopathy. *J. Neuropathol. Exp. Neurol.* 61:282–293.
- Thomas, T., Thomas, G., McLendon, C., Sutton, T., and Mullan, M. (1996). β -Amyloid-mediated vasoactivity and vascular endothelial damage. *Nature* 380:168–171.
- Tokuda, T., Calero, M., Matsubara, E., Vidal, R., Kumar, A., Permanne, B., Zlokovic, B.V., Smith, J.D., LaDu, M.J., Rostagno, A., Frangione, B., and Ghiso, J. (2000). Lipidation of apolipoprotein E influences its isoform-specific interaction with Alzheimer's amyloid β peptides. *Biochem. J.* 348:359–365.
- Tomiyama, T., Corder, E.H., and Mori, H. (1999). Molecular pathogenesis of apolipoprotein E-mediated amyloidosis in late-onset Alzheimer's disease. *Cell. Mol. Life Sci.* 56:268–279.

- Trigatti, B.L., Krieger, M., and Rigotti, A. (2003). Influence of the HDL receptor SR-BI on lipoprotein—metabolism and atherosclerosis. *Arterioscler. Thromb.Vasc. Biol.* 23:1732–1738.
- Tsuruta, J.K., Wong, K., Fritz, I.B., and Griswold, M.D. (1990). Structural-analysis of sulfated glycoprotein-2 from amino-acid-sequence—relationship to clusterin and serum protein-40,40. *Biochem. J.* 268:571–578.
- Van Dorpe, J., Smeijers, L., Dewachter, I., Nuyens, D., Spittaels, K., Van Den Haute, C., Mercken, M., Moechars, D., Laenen, I., Kuiperi, C., Bruynseels, K., Tesseur, I., Loos, R., Vanderstichele, H., Checler, F., Sciot, R., and Van Leuven, F. (2000). Prominent cerebral amyloid angiopathy in transgenic mice overexpressing the London mutant of human APP in neurons. *Am. J. Pathol.* 157:1283–1298.
- Van Everbroeck, B., Croes, E.A., Pals, P., Dermaut, B., Jansen, G., van Duijn, C.M., Cruts, M., Van Broeckhoven, C., Martin, J.J., and Cras, P. (2001). Influence of the prion protein and the apolipoprotein E genotype on the Creutzfeldt-Jakob disease phenotype. *Neurosci. Lett.* 313:69–72.
- Verbeek, M.M., Otte-Holler, I., Veerhuis, R., Ruiter, D.J., and De Waal, R.M.W. (1998). Distribution of A beta-associated proteins in cerebrovascular amyloid of Alzheimer's disease. *Acta Neuropathol.* 96:628–636.
- Vidal, J., Verchere, C.B., Andrikopoulos, S., Wang, F., Hull, R.L., Cnop, M., Olin, K.L., LeBoeuf, R.C., O'Brien, K.D., Chait, A., and Kahn, S.E. (2003). The effect of apolipoprotein E deficiency on islet amyloid deposition in human islet amyloid polypeptide transgenic mice. *Diabetologia* 46:71–79.
- Weisgraber, H.H. (1994). Apolipoprotein-e—structure—function-relationships. *Adv. Protein Chem.* 45:249–302.
- Wengenack, T.M., Curran, G.L., and Poduslo, J.F. (2000). Targeting Alzheimer amyloid plaques in vivo. *Nat. Biotechnol.* 18:868–872.
- Westermarck, P., Mucchiano, G., Marthin, T., Johnson, K.H., and Sletten, K. (1995). Apolipoprotein A1-derived amyloid in human aortic atherosclerotic plaques. *Am. J. Pathol.* 147:1186–1192.
- Wirths, O., Multhaup, G., Czech, C., Blanchard, V., Moussaoui, S., Tremp, G., Pradier, L., Beyreuther, K., and Bayer, T.A. (2001). Intraneuronal A beta accumulation precedes plaque formation in beta-amyloid precursor protein and presenilin-1 double-transgenic mice. *Neurosci. Lett.* 306:116–120.
- Wisniewski, H.M., Sadowski, M., Jakubowska-Sadowska, K., Tarnawski, M., and Wegiel, J. (1998). Diffuse, lake-like amyloid-beta deposits in the paraventricular layer of the presubiculum in Alzheimer disease. *J. Neuropathol. Exp. Neurol.* 57:674–683.
- Wisniewski, T., and Frangione, B. (1992). Apolipoprotein E: a pathological chaperone protein in patients with cerebral and systemic amyloid. *Neurosci. Lett.* 135:235–238.
- Wisniewski, T., Ghiso, J., and Frangione, B. (1991). Peptides homologous to the amyloid protein of Alzheimer's disease containing a glutamine for glutamic acid substitution have accelerated amyloid fibril formation. *Biochem. Biophys. Res. Commun.* 179:1247–1254.
- Wisniewski, T., Castaño, E.M., Ghiso, J., and Frangione, B. (1993a). Cerebrospinal fluid inhibits Alzheimer beta-amyloid fibril formation in vitro. *Ann. Neurol.* 34:631–633.
- Wisniewski, T., Golabek, A.A., Matsubara, E., Ghiso, J., and Frangione, B. (1993b). Apolipoprotein E: binding to soluble Alzheimer's beta-amyloid. *Biochem. Biophys. Res. Commun.* 192:359–365.
- Wisniewski, T., Castaño, E.M., Golabek, A.A., Vogel, T., and Frangione, B. (1994a). Acceleration of Alzheimer's fibril formation by apolipoprotein E in vitro. *Am. J. Pathol.* 145:1030–1035.
- Wisniewski, T., Ghiso, J., and Frangione, B. (1994b). Alzheimer's disease and soluble Aβ. *Neurobiol. Aging* 15: 143–152.
- Wisniewski, T., Golabek, A.A., Kida, E., Wisniewski, K.E., and Frangione, B. (1995a). Conformational mimicry in Alzheimer's disease. Role of apolipoproteins in amyloidogenesis. *Am. J. Pathol.* 147:238–244.
- Wisniewski, T., Lalowski, M., Golabek, A.A., Vogel, T., and Frangione, B. (1995b). Is Alzheimer's disease an apolipoprotein E amyloidosis? *Lancet* 345:956–958.
- Yamaguchi, H., Sugihara, S., Ogawa, A., Oshima, N., and Ihara, Y. (2001). Alzheimer beta amyloid deposition enhanced by ApoE epsilon 4 gene precedes neurofibrillary pathology in the frontal association cortex of nondemented senior subjects. *J. Neuropathol. Exp. Neurol.* 60:731–739.
- Yan, S.D., Chen, X., Fu, J., Chen, M., Zhu, H.J., Roher, A., Slattery, T., Zhao, L., Nagashima, M., Morser, J., Migheli, A., Nawroth, P., Stern, D., and Schmidt, A.M. (1996). RAGE and amyloid-β peptide neurotoxicity in Alzheimer's disease. *Nature* 382:685–691.
- Yan, S.D., Fu, J., Soto, C., Chen, X., Zhu, H.J., Al-Mohanna, F., Collison, K., Zhu, A.P., Stern, E., Saido, T., Tohyama, M., Ogawa, S., Roher, A., and Stern, D. (1997). An intracellular protein that binds amyloid-β peptide and mediates neurotoxicity in Alzheimer's disease. *Nature* 389:689–695.
- Zeidler, M., Stewart, G.E., Barraclough, C.R., Bateman, D.E., Bates, D., Burn, D.J., Colchester, A.C., Durward, W., Fletcher, N.A., Hawkins, S.A., Mackenzie, J.M., and Will, R.G. (1997). New variant Creutzfeldt-Jakob disease: neurological features and diagnostic tests. *Lancet* 350:903–907.

- Zerr, I., Helmhold, M., Poser, S., Armstrong, V.W., and Weber, T. (1996). Apolipoprotein E phenotype frequency and cerebrospinal fluid concentration are not associated with Creutzfeldt-Jakob disease. *Arch. Neurol.* 53:1233–1238.
- Zlokovic, B.V. (1996). Cerebrovascular transport of Alzheimer's amyloid β and apolipoproteins J and E: possible anti-amyloidogenic role of the blood–brain barrier. *Life Sci.* 59:1483–1497.
- Zlokovic, B.V., Ghiso, J., Mackic, J.B., McComb, J.G., Weiss, M.H., and Frangione, B. (1993). Blood–brain barrier transport of circulating Alzheimer's amyloid beta. *Biochem. Biophys. Res. Commun.* 197:1034–1040.
- Zlokovic, B.V., Martel, C.L., Mackic, J.B., Matsubara, E., Wisniewski, T., McComb, J.G., Frangione, B., and Ghiso, J. (1994). Brain uptake of circulating apolipoproteins J and E complexed to Alzheimer's amyloid beta. *Biochem. Biophys. Res. Commun.* 205:1431–1437.
- Zlokovic, B.V., Martel, C.L., Matsubara, E., McComb, J.G., Zheng, G., McCluskey, R.T., Frangione, B., and Ghiso, J. (1996). Glycoprotein 330/megalin: probable role in receptor-mediated transport of apolipoprotein J alone and in a complex with Alzheimer disease amyloid β at the blood–brain and blood–cerebrospinal fluid barriers. *Proc. Natl. Acad. Sci. USA* 93:4229–4234.
- Zlokovic, B.V., Yamada, S., Holtzman, D., Ghiso, J., and Frangione, B. (2000). Clearance of amyloid beta-peptide from brain: transport or metabolism? *Nat. Med.* 6:718.

Oxidative Stress and Protein Deposition Diseases

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1. Abstract

Despite divergent opinions on whether protein inclusions represent a protective or harmful mechanism in the progression of diseases such as Alzheimer's disease, Parkinson's disease, and prion diseases, oxidative and nitrative modifications may play a critical role in development of the diseases. Oxidative and nitrative modifications may occur early during the disease initiation, or later, compromising cellular functions once the inclusions have become sufficiently large at later stages of disease progression. These assertions are supported by exciting published but preliminary evidence, which requires extensive validation by experiments employing simple *in vitro* systems, cellular and animal models. Experiments in progress should inform us of the significance of oxidative and nitrative chemistries in the pathological mechanisms of diseases characterized by protein inclusions.

2. Introduction to Oxidative and Nitrative Stresses

Although oxygen metabolism is essential for life, it poses a potential threat to cells because of the formation of partially reduced reactive oxygen species. Oxygen is an ideal terminal acceptor of electrons, and generates the large thermodynamic force required for adenosine triphosphate (ATP) synthesis during aerobic metabolism. Oxidative phosphorylation carried out by large protein assemblies (respiratory complexes I, II, III, and IV) in the mitochondria is a major source of ATP. Electrons provided to the mitochondrial transport chain by reducing equivalents are transferred step by step through the respiratory complexes, some of which pump protons that form the proton-motive force powering the production of the ATP. The electrons ultimately reach the terminal oxidase of the complex, cytochrome *c* oxidase (complex IV), where four electrons reduce oxygen to water. Oxidative phosphorylation depends on this process of electron transfer, and inhibition of the process has detrimental effects on cells. The pesticide rotenone, which inhibits the transfer of electrons by the NADPH-Q oxidoreductase (complex I), and 3-nitropropionic acid, which inhibits the transfer of electrons by succinate-Q reductase (complex II), are both known to cause neuronal injury, and can reproduce features of Parkinson's disease in rodents and primates (Betarbet *et al.*, 2000; Lee *et al.*, 2002; Sherer *et al.*, 2002). Moreover, respiratory insufficiency of complex I has been detected in Parkinson's disease patients, suggesting that impaired mitochondrial respiration is a component of the disease pathology (Swerdlow *et al.*, 1996). Oxygen is also utilized by a number of other enzymatic processes such as monooxygenases, flavoproteins, cytochrome P450, and other

oxidases. Like the mitochondrial electron transport chain, electron transfer in these proteins is tightly coupled to avoid partial reduction of oxygen.

Partially reduced oxygen species are not strongly oxidizing on their own, but their reactivity and toxicity can be enhanced when combined with metal catalysts or other radical species. For example, hydrogen peroxide can form reactive hydroxyl radicals in the presence of redox-active iron that can cause damage to a wide range of biomolecules. Nitric oxide can react with superoxide to form peroxynitrite at a rate that is diffusion limited (Beckman *et al.*, 1990). Peroxynitrite can directly oxidize thiols and interact with metal centers or upon protonation, homolytic cleavage produces secondary radicals hydroxyl radical and nitrogen dioxide (Radi *et al.*, 2001). Peroxynitrite also reacts rapidly with carbon dioxide to produce carbonate radical and nitrogen dioxide (Radi *et al.*, 2001). Nitric oxide readily shifts to higher oxidation states, such as nitrite, which can also provide a substrate for peroxidase enzymes. In conjunction with hydrogen peroxide, peroxidases can generate nitrogen dioxide radical that can oxidize and nitrate proteins and lipids (Brennan *et al.*, 2002; Zhang *et al.*, 2002). Nitrite can also form nitrous acid, which is significantly less reactive than peroxynitrite and nitrogen dioxide, but can still modify biologic macromolecules (Ischiropoulos, 1998). These various species of reactive oxygen and nitrogen can modify proteins, lipids, and nucleic acids, possibly contributing to protein deposition in neurodegenerative disorders (Lyras *et al.*, 1998; Ischiropoulos and Beckman, 2003).

Determining the oxidative burden in human subjects and animal models is limited by the evanescent reactive nature of these species. Hence, evidence for their existence and role in disease comes from the detection of stable products formed after reactions with cellular components. Proteins are major targets for oxidation, and although every amino acid in proteins can be oxidized, certain amino acids such as cysteine, methionine, histidine, tryptophan, and tyrosine residues are more sensitive to oxidative modifications. Methionine residues are especially prone to two-electron oxidation, leading to the formation of the methionine sulfoxide. The discovery of methionine sulfoxide reductase, an enzyme that specifically repairs this oxidative modification, indicates that this residue is a target for oxidative modification *in vivo* (Moskovitz *et al.*, 2001). Tyrosine residues are also important targets for both reactive oxygen and nitrogen species. One-electron oxidation of the phenol group leads to a relatively stable, carbon centered radical, which can react with another tyrosyl radical forming dityrosine crosslinked protein dimers, trimers, and oligomers (Souza *et al.*, 2000). The reaction of tyrosine residues with reactive nitrogen species generates 3-nitrotyrosine, a unique signature protein modification (Ischiropoulos, 1998). Nitration of tyrosine residues in proteins could be derived *in vivo* by at least three reactive pathways: (1) peroxidase-catalyzed oxidation of nitrite to nitrogen dioxide; (2) formation of nitrous acid by the acidification of nitrite; or (3) formation of nitrogen dioxide and carbonate radical from the peroxynitrite-carbon dioxide intermediate. Carbonate radicals contribute to nitration by promoting one-electron abstraction rather than addition, leading to the formation of carbon-centered radicals, which readily reacts with nitrogen dioxide and thus favor nitration in certain conditions (Radi *et al.*, 2001). Despite the reactive nature of the nitrating agents, protein tyrosine nitration is a rather selective and specific process, as nitrated proteins are localized only at sites of injury and only few proteins within the injured tissue are modified in human diseases (Ischiropoulos, 1998).

Another commonly employed assay for oxidation damage involves the detection of reactive protein carbonyls. The formation of reactive carbonyls on proteins could result from several distinct biochemical pathways (Stadtman, 1992; Shacter *et al.*, 1994): (1) direct oxidation of amino acid residues such as arginine, histidine, and proline; (2) Michael-type addition to lysine or reduced cysteine of reactive bifunctional aldehydes generated by oxidation of polyunsaturated fatty acids; and (3) Schiff base formation and Amidori rearrangement of an oxidized sugar. All of these biochemical

pathways indicate either a direct amino acid oxidation or indirect oxidation, and thus, a potential direct correlation with the magnitude of oxidative stress. Other oxidative posttranslational modifiers known as advanced glycosylation end products (AGEs), are generated by oxidation of alpha-ketoaldehydes such as methylglyoxal. AGEs can form covalent adducts to lysine and arginine residues of proteins and have been shown to alter enzymatic functions of proteins involved in neurodegenerative processes as well as decrease protein degradation (Arai *et al.*, 1987; Brownlee *et al.*, 1983). In addition to oxidized proteins and carbohydrates, oxidized lipid products also mark the presence of oxidants. The most notable of these is 4-hydroxy-2-nonenal (4-HNE), a reactive aldehyde generated by oxidation of ν -6 unsaturated phospholipids within cellular membranes (Uchida and Stadtman, 1992; Matson *et al.*, 1997; Ando *et al.*, 1998; Perez *et al.*, 2000).

However, cells are endowed with an array of detoxifying enzymes, such as the cytosolic Cu–Zn superoxide dismutase (SOD1), catalase, glutathione peroxidase and reductase, as well as free radical scavengers such as glutathione and ascorbate. Within mitochondria, Mn superoxide dismutase (SOD2) and peroxidases are strategically located to remove partially reduced species of oxygen. However, this delicate balance between oxidants and antioxidants can be disrupted during pathological states by overproduction of oxidants, diminished antioxidant function, or a combination of both. Extensive reduction of oxygen to superoxide and hydrogen peroxide coupled with the inactivation of defense systems could result in a significant augmentation of oxidants that exceed the cellular antioxidant capacity. Once this occurs, macromolecules such as proteins, lipids, and DNA become oxidized. The oxidized macromolecules can undergo repair, or may be degraded. Proteasomal and other protein degrading mechanisms remove oxidatively modified proteins, and several DNA repair mechanisms correct oxidative lesions in DNA. A failure in these repair processes in conjunction with a persistent oxidation of macromolecules can also compromise cellular function, ultimately resulting in cellular death.

3. Oxidative and Nitritative Stresses in Neurodegenerative Diseases with Protein Deposits

Alzheimer's disease, Parkinson's disease, and other neurodegenerative disorders are characterized by the intracellular and/or extracellular presence of filamentous, proteinaceous deposits in the nervous system. Central to many investigations is the hypothesis that formation of these deposits is an important pathogenic event. Many studies have therefore focused on understanding the nature of aberrant protein aggregation and the role of this process in compromised cell function and cell death. Because of the clear association of oxidative stress with many protein deposition diseases, it has been postulated that oxidative and nitritative posttranslational modifications to proteins play a direct role in initiation, propagation, or further stabilization of the aggregation process (summarized in scheme, see **Figure 7-1; see color insert**). For example, protein oxidation may result in the initiation of aggregation by altering the conformation of the protein to a configuration that is more likely to aggregate or by generating protein dimers through disulfide or dityrosine linkage. These dimers can act as "seeds" to initiate the nucleation-dependent aggregation process of proteins (α -synuclein is an example discussed below). Oxidative and nitritative modified proteins may also contribute to the propagation of aggregation by interfering with the degradation of proteins by the proteolytic machinery of the cells. Alternatively, these modifications may act further downstream in the aggregation process by stabilizing pre-formed filaments. Evidence for oxidative and nitritative chemistry in both human neuropathology and accompanying animal and cell models is reviewed below, with emphasis

on the effects these chemistries have on protein aggregation in Alzheimer's disease, transmissible spongiform encephalopathies, and Parkinson's disease and related synucleinopathies.

3.1. Alzheimer's Disease

It has been well established that oxidative stress is associated with Alzheimer's disease (AD). Tau and A β proteins are primary components of the filamentous lesions in AD, and association of these lesions with redox-active iron as well as other transition metals has been shown (Good *et al.*, 1992; Smith *et al.*, 1997a; Lovell *et al.*, 1998; Honda *et al.*, 2004). Other post mortem analyses of brain tissue from AD patients reveal differential distribution of proteins involved in iron regulation, transferrin, and ferritin (Connor *et al.*, 1992), suggesting an imbalance in iron homeostasis and metabolism. Various markers of oxidative stress, such as AGEs, are also evident in AD. AGEs have been identified within the neurofibrillary tangles of Alzheimer's disease brain, and *in vitro* systems have shown that AGE-modified tau can induce oxidative stress in neuroblastoma cells (Yan *et al.*, 1994). In addition, it was recently discovered that expression of glyoxalase I, an enzyme that prevents the formation of AGEs, is upregulated in transgenic mice expressing a disease-associated mutant tau (Chen *et al.*, 2004), possibly representing a compensatory response to AGE formation. Although some degree of increased oxidative stress occurs in both normal aging and AD, as demonstrated by increased protein carbonyls and 4-HNE protein adducts (Ando, 1998; Aksenov *et al.*, 2001), studies show that in AD patients, some of these oxidative markers are increased within specific brain areas more prominently affected by the disease, such as the hippocampus (Aksenov *et al.*, 2001). These modifications can lead to alterations in protein function and turnover. For example, glutamine synthetase, an enzyme that mediates glutamate toxicity by converting glutamate to glutamine in astrocytes, is a specific target of protein carbonyl formation and shows decreased activity in Alzheimer's disease (Smith *et al.*, 1991; Butterfield *et al.*, 1997; Castegna *et al.*, 2002). Detection of proteins modified by tyrosine nitration is among the earliest markers of oxidative stress in the Alzheimer's disease brain (Smith *et al.*, 1997b; Hensley *et al.*, 1998). The relevance of this modification in protein deposition and neurodegeneration of Alzheimer's disease will be discussed below.

3.1.1. Oxidative Modifications of Tau Are Associated with Protein Deposition and Neurodegeneration

Although the aberrant phosphorylation of tau is considered a central factor in aggregation and decreased microtubule interactions, oxidation may also play an important role in this process. *In vitro* studies have shown that sulfhydryl oxidation of cysteine 322 in tau is responsible for dimerization and promotes paired helical filament formation. Other *in vitro* studies have demonstrated that tau oxidation might affect its physiological function. For example, exposure of tau to peroxynitrite and hydrogen peroxide results in disulfide linkages and decreased ability to bind and stabilize microtubules (Landino *et al.*, 2004). Furthermore, this effect can be reversed by treatment with the thioredoxin reductase system (Landino *et al.*, 2004). Showing that oxidation affect tau function (Schweers *et al.*, 1995).

Post mortem analysis of brain tissue from Alzheimer's disease and other neurodegenerative disorders also supports a role for tyrosine nitration in tau aggregation. Using antibodies that recognize a nitrated form of tau, immunohistochemical detection has shown that nitrated tau is a component of neurofibrillary tangles (Horiguchi *et al.*, 2003). Biochemical studies have confirmed the presence of nitrated tau in insoluble paired helical filament fractions of the diseased brain as well (Horiguchi *et al.*, 2003). Cell culture studies have demonstrated that tau is a target of nitration when cells are challenged with a nitrative insult (Horiguchi *et al.*, 2003). Although it is apparent that tau nitration

is associated with Alzheimer's disease and other tauopathies, it is unknown when in the filament-forming process this modification occurs. Studies have suggested that tau nitration may occur before the formation of mature filaments (Horiguchi *et al.*, 2003), but this has not been confirmed. Alternatively, dityrosine crosslinking could occur subsequent to filament assembly, acting to stabilize these structures. Although the functional consequences of tau nitration are unclear, this modification could cause a conformational change and liberate tau from microtubules, similar to phosphorylation, causing an increase in the pool of unbound tau that is more likely to aggregate. Whether this modification is causally related to the neurodegenerative processes of Alzheimer's disease and other tauopathies or merely is a reflection of secondary phenomena remains to be determined.

Another potential mechanism of tau aggregation is through the formation of AGEs. Histological analysis of Alzheimer's disease brain reveals that AGEs colocalize with neurofibrillary tangles (Yan *et al.*, 1994), suggesting that this modification may play a role in disease processes. Post mortem analysis of brain tissue suggests that glycation of tau occurs *in vivo* (Ledesma *et al.*, 1994) in the tubulin-binding domain (Ledesma *et al.*, 1995). *In vitro* studies have demonstrated that AGE-tau shows an increased propensity to aggregate into the formation of SDS-insoluble forms (Ledesma *et al.*, 1994, 1995). AGE modifications to other proteins can affect degradation rates (Brownlee *et al.*, 1983), and this modification may also slow the proteolytic degradation of tau, increasing its propensity to aggregate into paired helical filaments. In addition to direct effects on tau aggregation by glycation, these modifications may act indirectly. For example, AGE-tau has been shown to cause the release of 4-kD A β peptides, which may aid in the formation of extracellular A β aggregation (Yan *et al.*, 1995).

Other factors, including lipid oxidation, appear to induce tau aggregation *in vitro*. The process of lipid peroxidation has been also shown to induce tau aggregation. Specifically addition of 4-HNE, which can form adducts by a Michael-type addition to histidine residues (Uchida *et al.*, 1992), can induce aggregation of phosphorylated tau (Perez *et al.*, 2000). Studies have also shown that addition of 4-HNE to hippocampal cell cultures results in direct binding to tau and inhibition of tau dephosphorylation (Mattson *et al.*, 1997). This finding suggests an indirect effect of 4-HNE on tau aggregation since an increase in phosphorylated tau protein may contribute to paired helical formation by freeing tau from microtubules and increasing its propensity to aggregate. Overall, oxidative and nitrative processes may represent both early and late events in modifying tau and promoting the formation of tau deposits in the diseased brain.

3.2. Oxidative Protein Deposition in Transmissible Spongiform Encephalopathies

The neurodegenerative processes in Creutzfeld-Jakob disease and other transmissible spongiform encephalopathies are attributed to the isomerization of the mainly helical cellular prion protein (PrP^c) into a β -sheet-rich, protease-resistant form, known as scrapie prion protein (PrP^{Sc}). Many investigations have thus focused on the causes and consequences of this conformational change. Although the physiological function of PrP^c has not been defined, it may assist in balancing the redox potential of the cell. PrP^c can selectively bind copper (Hornshaw *et al.*, 1995; Brown *et al.*, 1997a; Stockel *et al.*, 1998), regulate the activity of SOD1 (Brown *et al.*, 1997b), and protect cells in culture from oxidative stress (Brown *et al.*, 1997c). Alteration of the cellular redox state can influence the conformation change from PrP^c to PrP^{Sc} through the formation of disulfide bridges. Disulfide bridge formation appears to be required for this conformational change (Herrmann and Caughey, 1998), and could affect the processing of PrP^c by affecting the glycosylation state of the protein (Capellari *et al.*, 1999). Under oxidative conditions, where formation of disulfide bridges is favored, the PrP^c protein is found to be predominantly in a nonglycosylated form (Capellari *et al.*, 1999). The ratio

of glycosylated to nonglycosylated forms of PrP^c may be an important factor in the conversion from PrP^c to PrP^{Sc}, with the nonglycosylated form favoring the protease resistant PrP^{Sc} conformation (Lehmann *et al.*, 1997). These studies demonstrate that the redox state of the cell may affect the conformation, solubility, and degradation of prion protein in transmissible spongiform encephalopathies.

3.3. Oxidative Stress and Synucleinopathies

α -Synuclein is a highly conserved 140-amino acid protein that is abundant in neurons, especially in presynaptic terminals (Clayton and George, 1999). Since the discovery of a genetic linkage of α -synuclein to familial Parkinson's disease (Polymeropoulos *et al.*, 1997; Kruger *et al.*, 1998), interest in this protein has grown enormously. α -Synuclein constitutes a major component of Lewy bodies and Lewy neurites, the intraneuronal inclusions characteristic of Parkinson's disease and related synucleinopathies (Baba *et al.*, 1998; Spillantini *et al.*, 1998). Electron microscopy reveals Lewy bodies as having a densely stained granular core surrounded by a halo of radiating α -synuclein filaments. The spontaneous formation of α -synuclein dimers appears to be the rate-limiting step of fibrillization, as these dimers act as nuclei for fibril assembly *in vitro* (Krishnan *et al.*, 2003). However, there is a considerable divergence in opinion regarding the toxic potential of α -synuclein fibrils. Fibrils may represent inert species and reflect secondary effects of the disease process rather than the primary means of pathogenesis. Alternatively, it has been proposed that an intermediate conformation between dimers and fibrils, termed protofibril, may be the proximal toxic intermediates (Conway *et al.*, 2001; Lashuel *et al.*, 2002). Microscopic examination of these α -synuclein protofibrils reveals a pore-like structure resembling the cytolytic β -barrel toxins formed by bacteria such as *Clostridium perfringens* (Lashuel *et al.*, 2002). Whether fibrils or protofibrils are central to the disease process remains to be determined, and the role of oxidative modifications to α -synuclein may be key to resolving this question.

Although the stage at which oxidative or nitrative modifications to α -synuclein may influence the disease process is not clear, involvement of these modified species has been demonstrated. Post mortem analysis of brain tissue reveals elevated levels of protein carbonyls and DNA oxidation products in the parietal and temporal cortex of dementia with Lewy body patients compared to control patients (Lyras *et al.*, 1998). In Parkinson's disease patients, decreased levels of glutathione and increased levels of free iron have been found in the substantia nigra pars compacta (SNc), along with evidence of oxidative damage to proteins, DNA, and lipids in this region of the brain (Lyras *et al.*, 1998; Andersen, 2003). Nitration of α -synuclein and its effects on fibril formation have become important areas of investigation since the discovery of nitrated α -synuclein in inclusions of patients with different synucleinopathies (Duda *et al.*, 2000; Giasson *et al.*, 2000). With the generation and employment of specific monoclonal antibodies generated against nitrated α -synuclein, nitrated α -synuclein has been identified as a component of Lewy bodies (Giasson *et al.*, 2000). Nitrated α -synuclein is found only in aggregated, insoluble form, but not in the soluble pool, strongly suggesting a link between nitration and aggregation (Giasson *et al.*, 2000). Incubation of α -synuclein with peroxide and divalent metal ions generates SDS-stable α -synuclein dimers and oligomers (Paik *et al.*, 1999, 2000; Norris *et al.*, 2003). Dityrosine crosslinking is evident in the dimers and oligomers, and α -synuclein lacks other residues such as cysteine or tryptophan that would be prone to oxidative or nitrative modifications. However, dityrosine crosslinks are not the only mechanism of dimer and oligomer formation (Souza *et al.*, 2000). α -Synuclein mutants with all four tyrosine residues substituted by phenylalanine can still form dimers after exposure to peroxide and copper, and this mutant protein is still able to form fibrils (Norris *et al.*, 2003). It remains to be determined which amino acid residues are responsible for dimer formation in this α -synuclein mutant. However, nitration of

tyrosine is the only oxidative posttranslational modification in the α -synuclein molecule that has been detected *in vivo*.

Despite substantial evidence of oxidative and nitrative damage in synucleinopathies, the direct effect these modifications have on the ability of α -synuclein to aggregate into amyloid-type inclusions is not well characterized. Exposure of α -synuclein to high concentrations of peroxide (1.2M) renders α -synuclein incapable of forming fibrils (Uversky *et al.*, 2002). Such harsh treatment results in oxidation of all four methionine residues to their respective sulfoxides, without significant oxidation of any other amino acid residue (Uversky *et al.*, 2002). However, milder treatments with lower concentrations of peroxide (300 μ M) in the presence of metal catalysts, have no effect on α -synuclein fibril formation (Norris *et al.*, 2003). In addition, some studies show that incubations of α -synuclein with redox active metals increase fibril formation (Ostrerova-Golts *et al.*, 2000; Uversky *et al.*, 2001; Yamin *et al.*, 2003a), suggesting that oxidative modifications may promote aggregation. These discrepancies may arise from dependence of fibrillization on the degree of oxidation. Studies using α -synuclein mutants reveal a linear relationship between the number of methionine residues present and the degree of inhibition of fibril formation after exposure to peroxide (Yamin *et al.*, 2003a). α -Synuclein mutants with all four methionine residues mutated are completely resistant to peroxide-mediated inhibition of fibrillization. Different oxidizing conditions may also create a spectrum of reactive species, leading to formation of a variety of modified α -synuclein species, which may differentially regulate the formation of fibrils.

Studies using α -synuclein-transfected HEK 293 cells show that simultaneous exposure to nitric oxide and superoxide-generating compounds leads to the formation of fibrillar intracellular α -synuclein inclusions with structure similar to those found *in vivo* (Paxinou *et al.*, 2001): demonstrating that reactive nitrogen species can cause protein aggregation in a cellular system. Exposure to either superoxide-generating compounds or nitric oxide alone did not lead to the formation of inclusions. Nitrated α -synuclein is present in these inclusions, suggesting a specific and causative role of nitration in α -synuclein deposition. In contrast with the *in vivo* data, studies *in vitro* show that nitration of α -synuclein with either peroxynitrite or tetranitromethane (TNM) inhibits the formation of fibrils (Souza *et al.*, 2000; Takahashi, 2002; Norris *et al.*, 2003; Yamin *et al.*, 2003b). These discrepancies may arise from the large bolus additions of TNM or peroxynitrite used to nitrate α -synuclein *in vitro*. *In vivo*, exposure to nitrating agents occurs in a steadily modulated flux, and studies have established that peroxynitrite administered in a steady flux yields more dityrosine crosslinked proteins than when the same amount is added in the form of bolus addition (Radi *et al.*, 2001). Therefore, the manner in which the nitrating agent is administered may affect the final result. Moreover, nitrating species such as TNM or peroxynitrite are strong oxidants, and will also cause oxidation of methionine residues. Treatment of α -synuclein with nitrating agents will generate a heterogeneous mixture composed of nitrated α -synuclein monomers, dimers, and oligomers, as well as nonnitrated and methionine oxidized proteins in different degrees. As stated above, these species may have profound and different effects on the process of fibril formation. Experiments that employ controlled chemical treatments, various α -synuclein mutants, and isolation of the different α -synuclein species are needed to elucidate the role of the oxidative and nitrative modifications on the formation of α -synuclein fibrils.

On the other hand, α -synuclein oligomers, which transiently appear during the process of fibril formation, can be stabilized under certain conditions, inhibiting the formation of fibrils. Incubation of human and mouse α -synuclein, which differ slightly in sequence, can stabilize oligomers, as can incubation with dopamine (Conway *et al.*, 2001; Li *et al.*, 2004). The inability of tyrosine to replicate the effect of dopamine indicates that the 3' hydroxyl group on the aromatic ring of dopamine is required, an observation corroborated by the ability of L-DOPA to replicate the effect of dopamine. Furthermore, scavengers of reactive species are capable of preventing the effect of dopamine in sta-

bilizing the oligomers, indicating that the interaction of α -synuclein with dopamine requires oxidation of the neurotransmitter (Conway *et al.*, 2001; Li *et al.*, 2004). The intimate association of α -synuclein and dopamine has also been suggested by studies using cell model systems. These studies reveal that expression of α -synuclein regulates both the expression and activity of proteins in the biosynthetic, metabolic pathways of dopamine, and neurotransmission (Abeliovich *et al.*, 2000; Masliah *et al.*, 2000; Tabrizi *et al.* 2000; Stefanis *et al.*, 2001; Lotharius and Brundin, 2002; Perez *et al.*, 2002; Xu *et al.* 2002). Despite significant divergence in opinions regarding the toxic potential of either protofibrils or fibrils, the initiation of the process could be sufficient to drive cellular dysfunction. Moreover, it remains unclear if the α -synuclein oligomers formed after dopamine treatments are similar in nature to those resulting from treatment with H_2O_2 and metals and additional structural studies characterizing these oligomeric species are needed.

4. Abbreviations

AD	Alzheimer's disease
AGE	Advanced glycosylation end products
4-HNE	4-Hydroxy-2-nonenal
PrP	Prion protein
SOD1	Cu-Zn superoxide dismutase
SOD2	Mn superoxide dismutase
TNM	Tetranitromethane

References

- Abeliovich, A., Schmitz, Y., Farinas, I., Choi-Lundberg, D., Ho, W.H., Catillo, P.E., Shinsky, N., Verdugo, J.M., Armanini, M., Ryan, A., Hynes, M., Phillips, H., Sulzer, D., and Rosenthal, A. (2000). Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. *Neuron* 25:239–252.
- Aksenov, M.Y., Aksenova, M.V., Butterfield, D.A., Geddes, J.W., and Markesbery, W.R. (2001). Protein oxidation in Alzheimer's disease. *Neuroscience* 103:373–383.
- Andersen, J.K. (2003). Paraquat and iron exposure as possible synergistic environmental risk factors in Parkinson's disease. *Neurotoxicol. Res.* 5:307–313.
- Ando, Y., Brannstrom, T., Uchida, K., Nyhlin, N., Nasman, B., Suhr, O., Yamashita, T., Olsson, T., El Salhy, M., Uchino, M., and Ando, M. (1998). Histochemical detection of 4-hydroxynonenal protein in Alzheimer amyloid. *J. Neurol. Sci.* 156:172–176.
- Arai, K., Maguchi, S., Fujii, S., Ishibashi, H., Oikawa, K., and Taniguchi, N. (1987). Glycation and inactivation of human Cu-Zn-superoxide dismutase. Identification of the in vitro glycosylated sites. *J. Biol. Chem.* 262:16969–16972.
- Baba, M., Nakajo, S., Tu, P.H., Tomita, T., Nakaya, K., Lee, V.M.-Y., Trojanowski, J.Q., and Iwatsubo, T. (1998). Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. *Am. J. Pathol.* 152:879–884.
- Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A., and Freeman, B.A. (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. USA* 87:1620–1624.
- Betarbet, R., Sherer, T.B., MacKenzie, G., Garcia-Osuna, M., Panov, A.V., and Greenamyre, J.T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat. Neurosci.* 3:1301–1306.
- Brennan, M.L., Wu, W., Fu, X., Shen, Z., Song, W., Frost, H., Vadseth, C., Narine, L., Lenkiewicz, E., Bochers, M.T., Lusis, A.J., Lee, J.J., Lee, N.A., Abu-Soud, H.M., Ischiropoulos, H., and Hazen, S. (2002). A tale of two controversies: defining the role of peroxidases in nitrotyrosine formation in vivo using eosinophil peroxidase and myeloperoxidase deficient mice; and the nature of peroxidase-generated reactive nitrogen species. *J. Biol. Chem.* 277:17415–17427.

- Brown, D.R., Qin, K., Herms, J.W., Madlung, A., Manson, J., Strome, R., Fraser, P.E., Kruck, T., von Bohlen, A., Schulz-Schaeffer, W., Giese, A., Westaway, D., and Kretzschmar, H. (1997a). The cellular prion protein binds copper in vivo. *Nature* 390:684–687.
- Brown, D.R., Schulz-Schaeffer, W.J., Schmidt, B., and Kretzschmar, H.A. (1997b). Prion protein-deficient cells show altered response to oxidative stress due to decreased SOD-1 activity. *Exp. Neurol.* 146:104–112.
- Brown, D.R., Schmidt, B., and Kretzschmar, H.A. (1997c). Effects of oxidative stress on prion protein expression in PC12 cells. *Int. J. Dev. Neurosci.* 15:961–972.
- Brownlee, M., Vlassara, H., and Cerami, A. (1983). Nonenzymatic glycosylation reduces the susceptibility of fibrin to degradation by plasmin. *Diabetes* 32:680–684.
- Butterfield, D.A., Hensley, K., Cole, P., Subramaniam, R., Aksenov, M., Aksenova, M., Bummer, P.M., Haley, B.E., and Carney, J.M. (1997). Oxidatively induced structural alteration of glutamine synthetase assessed by analysis of spin label incorporation kinetics: relevance to Alzheimer's disease. *J. Neurochem.* 68:2451–2457.
- Capellari, S., Zaidi, S.I., Urig, C.B., Perry, G., Smith, M.A., and Petersen, R.B. (1999). Prion protein glycosylation is sensitive to redox change. *J. Biol. Chem.* 274:34846–34850.
- Castegna, A., Aksenov, M., Aksenova, M., Thongboonkerd, V., Klein, J.B., Pierce, W.M., Booze, R., Markesbery, W.R., and Butterfield, D.A. (2002). Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part I: creatine kinase BB, glutamine synthase, and ubiquitin carboxy-terminal hydrolase L. *Free Radic. Biol. Med.* 33:562–571.
- Chen, F., Wollmer, M.A., Hoerndli, F., Munch, G., Kuhla, B., Rogaev, E.I., Tsolaki, M., Papassotiropoulos, A., and Gotz, J. (2004). Role for glyoxalase I in Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* 101:7687–7692.
- Clayton, D.F., and George, J.M. (1999). Synucleins in synaptic plasticity and neurodegenerative disorders. *J. Neurosci. Res.* 58:120–129.
- Connor, J.R., Menzies, S.L., St. Martin, S.M., and Mufson, E.J. (1992). A histochemical study of iron, transferrin, and ferritin in Alzheimer's diseased brains. *J. Neurosci. Res.* 31:75–83.
- Conway, K.A., Rochet, J.C., Bieganski, R.M., and Lansbury, P.T. (2001). Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct. *Science* 294:1346–1349.
- Duda, J.E., Giasson, B.I., Chen, Q., Gur, T.L., Hurtig, H.I., Stern, M.B., Gollomp, S.M., Ischiropoulos, H., Lee, V.M.-Y., and Trojanowski, J.Q. (2000). Widespread nitration of pathological inclusions in neurodegenerative synucleinopathies. *Am. J. Pathol.* 157:1439–1445.
- Giasson, B.I., Duda, J.E., Murray, I.V., Chen, Q., Souza, J.M., Hurtig, H.I., Ischiropoulos, H., Trojanowski, J.Q., and Lee, V.M.-Y. (2000). Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science* 290:985–989.
- Good, P.F., Perl, D.P., Bierer, L.M., and Schmeidler, J. (1992). Selective accumulation of aluminum and iron in the neurofibrillary tangles of Alzheimer's disease: a laser microprobe (LAMMA) study. *Ann. Neurol.* 31:286–292.
- Hensley, K., Maidt, M.L., Yu, Z., Sang, H., Markesbery, W.R., and Floyd, R.A. (1998). Electrochemical analysis of protein nitrotyrosine and dityrosine in the Alzheimer brain indicates region-specific accumulation. *J. Neurosci.* 18:8126–8132.
- Herrmann, L.M., and Caughey, B. (1998). The importance of the disulfide bond in prion protein conversion. *Neuroreport* 9:2457–2461.
- Honda, K., Casadesus, G., Petersen, R.B., Perry, G., and Smith, M.A. (2004). Oxidative stress and redox-active iron in Alzheimer's disease. *Ann. N. Y. Acad. Sci.* 1012:179–182.
- Horiguchi, T., Uryu, K., Giasson, B.I., Ischiropoulos, H., Lightfoot, R., Bellmann, C., Richter-Landsberg, C., Lee, V.M., and Trojanowski, J.Q. (2003). Nitration of tau protein is linked to neurodegeneration in tauopathies. *Am. J. Pathol.* 163:1021–1031.
- Hornshaw, M.P., McDermott, J.R., and Candy, J.M. (1995). Copper binding to the N-terminal tandem repeat regions of mammalian and avian prion protein. *Biochem. Biophys. Res. Commun.* 207:621–629.
- Ischiropoulos, H. (1998). Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch. Biochem. Biophys.* 356:1–11.
- Ischiropoulos, H., and Beckman, J.S. (2003). Oxidative stress and nitration in neurodegeneration: cause, effect, or association? *J. Clin. Invest.* 111:163–169.
- Krishnan, S., Chi, E.Y., Wood, S.J., Kendrick, B.S., Li, C., Garzon-Rodriguez, W., Wypych, J., Randolph, T.W., Narhi, L.O., Biere, A.L., Citron, M., and Carpenter, J.F. (2003). Oxidative dimer formation is the critical rate-limiting step for Parkinson's disease alpha-synuclein fibrillogenesis. *Biochemistry* 42:829–837.
- Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J.T., Schols, L., and Riess, O. (1998). Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nat. Genet.* 18:106–108.

- Landino, L.M., Skreslet, T.E., and Alston, J.A. (2004). Cysteine oxidation of tau and microtubule-associated protein-2 by peroxynitrite: modulation of microtubule assembly kinetics by the thioredoxin reductase system. *J. Biol. Chem.* 279:35101–35105.
- Lashuel, H.A., Hartley, D., Petre, B.M., Walz, T., and Lansbury, P.T. (2002). Neurodegenerative disease: amyloid pores from pathogenic mutations. *Nature* 418:291.
- Ledesma, M.D., Bonay, P., Colaco, C., and Avila, J. (1994). Analysis of microtubule-associated protein tau glycation in paired helical filaments. *J. Biol. Chem.* 269:21614–21619.
- Ledesma, M.D., Bonay, P., and Avila, J. (1995). Tau protein from Alzheimer's disease patients is glycated at its tubulin-binding domain. *J. Neurochem.* 65:1658–1664.
- Lee, H.J., Shin, S.Y., Choi, C., Lee, Y.H., and Lee, S.J. (2002). Formation and removal of alpha-synuclein aggregates in cells exposed to mitochondrial inhibitors. *J. Biol. Chem.* 277:5411–5417.
- Lehmann, S., and Harris, D.A. (1997). Blockade of glycosylation promotes acquisition of scrapie-like properties by the prion protein in cultured cells. *J. Biol. Chem.* 272:21479–21487.
- Li, J., Zhu, M., Manning-Bog, A.B., Di Monte, D.A., and Fink, A.L. (2004). Dopamine and L-dopa disaggregate amyloid fibrils: implication for Parkinson's and Alzheimer's disease. *FASEB J.* 18:962–964.
- Lotharius, J., and Brundin, P. (2002). Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein. *Nat. Rev. Neurosci.* 3:1–11.
- Lovell, M.A., Robertson, J.D., Teesdale, W.J., Campbell, J.L., and Markesbery, W.R. (1998). Copper, iron, and zinc in Alzheimer's disease senile plaques. *J. Neurol. Sci.* 158:47–52.
- Lyras, L., Perry, R.H., Perry, E.K., Ince, P.G., Jenner, A., Jenner, P., and Halliwell, B. (1998). Oxidative damage to proteins, lipids, and DNA in cortical brain regions from patients with dementia with Lewy bodies. *J. Neurochem.* 71:302–312.
- Masliah, E., Rockenstein, E., Veinbergs, I., Mallory, M., Hashimoto, M., Takeda, A., Sagara, Y., Sisk, A., and Mucke, L. (2000). Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. *Science* 287:1265–1269.
- Mattson, M.P., Fu, W., Waeg, G., and Uchida, K. (1997). 4-Hydroxynonenal, a product of lipid peroxidation, inhibits dephosphorylation of the microtubule-associated protein tau. *Neuroreport* 8:2275–2281.
- Moskovitz, J., Bar-Noy, S., Williams, W.M., Requena, J., Berlett, B.S., and Stadtman, E.R. (2001). Methionine sulfoxide reductase (MsrA) is a regulator of antioxidant defense and lifespan in mammals. *Proc. Natl. Acad. Sci. USA* 98:12920–12925.
- Norris, E.H., Giasson, B.I., Ischiropoulos, H., and Lee, V.M.-Y. (2003). Effects of oxidative and nitrative challenges on alpha-synuclein fibrillogenesis involve distinct mechanisms of protein modifications. *J. Biol. Chem.* 278:27230–27240.
- Ostrerova-Golts, N., Petrucelli, L., Hardy, J., Lee, J.M., Farrer, M., and Wolozin, B. (2000). The A53T alpha-synuclein mutation increases iron-dependent aggregation and toxicity. *J. Neurosci.* 20:6048–6054.
- Paik, S.R., Shin, H.J., Lee, J.H., Chang, C.S., and Kim, J. (1999). Copper(II)-induced self-oligomerization of alpha-synuclein. *Biochem. J.* 340:821–828.
- Paik, S.R., Shin, H.J., and Lee, J.H. (2000). Metal-catalyzed oxidation of alpha-synuclein in the presence of Copper (II) and hydrogen peroxide. *Arch. Biochem. Biophys.* 378:269–277.
- Paxinou, E., Chen, Q., Weisse, M., Giasson, B.I., Norris, E.H., Rueter, S.M., Trojanowski, J.Q., Lee, V.M.-Y., and Ischiropoulos, H. (2001). Induction of alpha-synuclein aggregation by intracellular nitrative insult. *J. Neurosci.* 21:8053–8061.
- Perez, M., Cuadros, R., Smith, M.A., Perry, G., and Avila, J. (2000). Phosphorylated, but not native, tau protein assembles following reaction with the lipid peroxidation product, 4-hydroxy-2-nonenal. *FEBS. Lett.* 486:270–274.
- Perez, R.G., Waymire, J.C., Lin, E., Liu, J.J., Guo, F. and Zigmond, M.J. (2002). A role for alpha-synuclein in the regulation of dopamine biosynthesis. *J. Neurosci.* 22:3090–3099.
- Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E.S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W.G., Lazzarini, A.M., Duvoisin, R.C., Di Iorio, G., Golbe, L.I., and Nussbaum, R.L. (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 276:2045–2047.
- Radi, R., Peluffo, G., Alvarez, M.-N., Naviliat, M., and Cayota, A. (2001). Unraveling peroxynitrite formation in biological systems. *Free Radic. Biol. Med.* 30:463–488.
- Schweers, O., Mandelkow, E.M., Biernat, J., and Mandelkow, E. (1995). Oxidation of cysteine-322 in the repeat domain of microtubule-associated protein tau controls the in vitro assembly of paired helical filaments. *Proc. Natl. Acad. Sci. USA.* 92:8463–8467.

- Shacter, E., Williams, J.A., Lim, M., and Levine, R.L. (1994). Differential susceptibility of plasma proteins to oxidative modification: examination by Western blot immunoassay. *Free Radic. Biol. Med.* 17:429–437.
- Sherer, T.B., Betarbet, R., Stout, A.K., Lund, S., Baptista, M., Panov, A.V., Cookson, M.R., and Greenamyre, J.T. (2002). An in vitro model of Parkinson's disease: linking mitochondrial impairment to altered alpha-synuclein metabolism and oxidative damage. *J. Neurosci.* 22:7006–7015.
- Smith, C.D., Carney, J.M., Starke-Reed, P.E., Oliver, C.N., Stadtman, E.R., Floyd, R.A., and Markesbery, W.R. (1991). Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. *Proc. Natl. Acad. Sci. USA* 88:10540–10543.
- Smith, M.A., Harris, P.L.R., Sayre, L.M., and Perry, G. (1997a). Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. *Proc. Natl. Acad. Sci. USA* 94:9866–9868.
- Smith, M.A., Richey Harris, P.L., Sayre, L.M., Beckamn, J.S., and Perry, G. (1997b). Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J. Neurosci.* 17:2653–2657.
- Souza, J.M., Giasson, B.I., Chen, Q., Lee, V.M.-Y., and Ischiropoulos, H. (2000). Dityrosine cross-linking promotes formation of stable alpha-synuclein polymers. Implication of nitrate and oxidative stress in the pathogenesis of neurodegenerative synucleinopathies. *J. Biol. Chem.* 275:18344–18349.
- Swerdlow, R.H., Parks, J.K., Miller, S.W., Tuttle, J.B., Trimmer, P.A., Sheehan, J.P., Bennett, J.P., Jr., Davis, R.E., and Parker, W.D., Jr. (1996). Origin and functional consequences of the complex I defect in Parkinson's disease. *Ann. Neurol.* 40:663–671.
- Spillantini, M.G., Crowther, R.A., Jakes, R., Hasegawa, M., and Goedert, M. (1998). alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. *Proc. Natl. Acad. Sci. USA* 95:6469–6473.
- Stadtman, E.R. (1992). Protein oxidation and Aging. *Science* 257:1220–1224.
- Stefanis, L., Larsen, K.E., Rideout, H.J., Sulzer, D., and Greene, L.A. (2001). Expression of A53T mutant but not wild-type alpha-synuclein in PC12 cells induces alterations of the ubiquitin-dependent degradation system, loss of dopamine release, and autophagic cell death. *J. Neurosci.* 21:9549–9560.
- Stockel, J., Safar, J., Wallace, A.C., Cohen, F.E., and Prusiner, S.B. (1998). Prion protein selectively binds copper(II) ions. *Biochemistry* 37:7185–7193.
- Tabrizi, S.J., Orth, M., Wilkinson, J.M., Taanman, J.W., Warner, T.T., Cooper, J.M., and Schapira, A.H. (2000). Expression of mutant alpha-synuclein causes increased susceptibility to dopamine toxicity. *Hum. Mol. Genet.* 9:2683–2689.
- Takahashi, T., Yamashita, H., Nakamura, T., Nagano, Y., and Nakamura, S. (2002). Tyrosine 125 of alpha-synuclein plays a critical role for dimerization following nitrate stress. *Brain. Res.* 938:73–80.
- Uchida, K., and Stadtman, E.R. (1992). Modification of histidine residues in proteins by reaction with 4-hydroxy nonenal. *Proc. Natl. Acad. Sci. USA* 89:4544–4548.
- Uversky, V.N., Li, J., and Fink, A.L. (2001). Metal-triggered structural transformations, aggregation, and fibrillation of human alpha-synuclein. A possible molecular link between Parkinson's disease and heavy metal exposure. *J. Biol. Chem.* 276:44284–44296.
- Uversky, V.N., Yamin, G., Souillac, P.O., Goers, J., Glaser, C.B., and Fink, A.L. (2002). Methionine oxidation inhibits fibrillation of human alpha-synuclein in vitro. *FEBS Lett.* 517:239–244.
- Yamin, G., Glaser, C.B., Uversky, V.N., and Fink, A.L. (2003a). Certain metals trigger fibrillation of methionine-oxidized alpha-synuclein. *J. Biol. Chem.* 278:27630–27635.
- Yamin, G., Uversky, V.N., and Fink, A.L. (2003b). Nitration inhibits fibrillation of human alpha-synuclein in vitro by formation of soluble oligomers. *FEBS Lett.* 542:147–152.
- Yan, S.D., Chen, X., Schmidt, A.M., Brett, J., Godman, G., Zou, Y.S., Scott, C.W., Caputo, C., Frappier, T., Smith, M.A. (1994). Glycated tau protein in Alzheimer disease: a mechanism for induction of oxidant stress. *Proc. Natl. Acad. Sci. USA* 91:7787–7791.
- Yan, S.D., Yan, S.F., Chen, X., Fu, J., Chen, M., Kuppusamy, P., Smith, M.A., Perry, G., Godman, G.C., and Nawroth P. (1995). Non-enzymatically glycated tau in Alzheimer's disease induces neuronal oxidant stress resulting in cytokine gene expression and release of amyloid beta-peptide. *Nat. Med.* 1:693–699.
- Xu, J., Kao, S.Y., Lee, F.J., Song, W., Jin, L.W., and Yankner, B.A. (2002). Dopamine-dependent neurotoxicity of alpha-synuclein: a mechanism for selective neurodegeneration in Parkinson disease. *Nat. Med.* 8:600–606.
- Zhang, R., Brennan, M.L., Shen, Z., MacPherson, J.C., Schmitt, D., Molenda, C.E., and Hazen, S.L. (2002). Myeloperoxidase functions as a major enzymatic catalyst for initiation of lipid peroxidation at sites of inflammation. *J. Biol. Chem.* 277:46116–46122.

Chaperone and Conformational Disorders

Chaperone Suppression of Aggregated Protein Toxicity

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1. Abstract

Overwhelming experimental evidence supports the hypothesis that molecular chaperones are critical modulators of protein aggregation and toxicity in a number of protein misfolding diseases. However, the mechanism by which chaperone activity facilitates neuroprotection remains poorly understood. Early intermediates in the assembly process of A β aggregates have been found to be potent neurotoxins *in vivo*, and it is likely that prefibrillar intermediates of other disease proteins may have similar pathogenic effects. Accordingly, a key step in the pathogenesis of the various proteinopathies may stem from the aberrant interactions of altered protein conformations or prefibrillar intermediates with key cellular proteins, effectively sequestering their activity and triggering a cascade of events that culminates in neuronal dysfunction prior to the appearance of inclusions. The vast majority of animal studies have shown that chaperones facilitate neuroprotection in the absence of a visible effect on inclusion formation, suggesting that protective interactions may occur at the level of prefibrillar aggregation intermediates, or by preventing conformational changes that precede the formation of aggregation intermediates. It will be important to develop techniques that enable *in vivo* detection of early aggregation intermediates for the various protein misfolding diseases and determine how interaction of these intermediates with other cellular proteins, such as the molecular chaperones, alters pathogenesis. Ultimately, it is necessary to understand how the various components of the protein quality proteome work together to regulate the toxicity of misfolded proteins. Effective therapies will likely require the simultaneous modulation of numerous components of the cellular quality control apparatus, and the molecular chaperones will play a key role in these types of approaches. Because the molecular chaperones provide a first line of defense against misfolded proteins, and are likely to function at the earliest stages of disease pathogenesis, they are a particularly exciting prospect for therapeutic intervention.

2. Protein Folding and Misfolding

A fundamental principle of biology states that the amino acid sequence of a protein contains all of the information necessary to dictate proper folding into a functional, three-dimensional structure (Anfinsen, 1973). *In vitro* refolding experiments have shown (Mitraki *et al.*, 1987), however, that when examining proteins of increasing size and/or complexity, such as multidomain proteins, the efficiency of spontaneous refolding decreases. Misfolded proteins can form when regions of a polypeptide chain that do not interact in the native state form stable complexes and prevent the protein

from reaching a functional three-dimensional structure. Because misfolded intermediates expose hydrophobic amino acid residues and segments of unstructured polypeptide backbone that are normally buried in the core of the protein, they are very prone to aggregation. *In vivo*, the highly crowded macromolecular environment promotes protein misfolding and aggregation, and as a result, protein folding is typically not a spontaneous process (Ellis, 2001). Living systems from archaea to eukaryotes have thus evolved a highly conserved class of proteins, the molecular chaperones, which by preventing inappropriate interactions within and between nonnative polypeptides, enhance the efficiency of *de novo* protein folding and promote the refolding of preexistent proteins that have become misfolded as a result of cell stress (Hartl and Hayer-Hartl, 2002). The chaperones interact transiently with unfolded proteins, and rather than increasing the rate of folding, increase the yield of functional protein. Chaperones do not, however, provide steric information, and are not present in the folded protein (Ellis and Hartl, 1999). In addition to protein folding, the molecular chaperones play an important role in several other cellular processes, including protein targeting, transport, degradation, and signal transduction (Hartl and Hayer-Hartl, 2002).

3. Cellular Quality Control

3.1. The Molecular Chaperones

A clear indication of the importance of the molecular chaperones in maintaining cellular homeostasis comes from the fact that in response to even a mild elevation in temperature there is a dramatic decrease in the synthesis of most proteins, and a robust increase in the synthesis of several chaperone proteins, termed heat-shock proteins (Hsp). This protective feedback system is activated under numerous other conditions including ischemia, oxidation, ultraviolet radiation, and exposure to ethanol, and thus has been termed the stress response (Lindquist, 1986; Smith *et al.*, 1998). The major Hsp families, named according to their approximate molecular weight (in kilodaltons) are Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and the small Hsp family (<40kDa).

Hsp100 family member Hsp104 plays an important role in thermotolerance in yeast where it seems to regulate both protein aggregation and disaggregation, but as of yet no mammalian homolog has been identified (Lindquist and Kim, 1996; Glover and Lindquist, 1998; Shorter and Lindquist, 2004). Hsp90, which is expressed at low levels and seems to play a minor role in prokaryotes, is abundantly expressed in the eukaryotic cytoplasm, where it is an essential component (Smith *et al.*, 1998). Although Hsp90 does not seem critical for nascent chain folding, it is able to hold nonnative proteins in a stable state. Furthermore, Hsp90 plays a critical role in regulating various signaling proteins, as evidenced by their well-characterized interactions with various steroid hormone receptors and tyrosine kinases (Citri *et al.*, 2004). Hsp60 family member GroEL, which is arguably the best-characterized chaperone, in cooperation with its cochaperone GroES (an Hsp10), serves an important function in prokaryotes to facilitate the folding of nascent chains downstream of DnaK (an Hsp70) and DnaJ (an Hsp40) (Hartl, 1996). In eukaryotic cells, the closest Hsp60 homologs are found in mitochondria and chloroplasts, presumably derived from prokaryotic organelles. Accordingly, Hsp60 family members have limited substrates in the eukaryotic cytosol (Hartl and Hayer-Hartl, 2002). Similar to the Hsp60 family, Hsp70 chaperones interact with nonnative proteins in an ATP-dependent manner, binding and releasing substrates as a result of conformational changes associated with ATP hydrolysis (Bukau and Horwich, 1998). Yeast have seven major cytosolic Hsp70 proteins, termed Ssa1–Ssa4 and Ssb1–Ssb2. In humans, 11 genes encoding Hsp70 family members have been identified (Tavaria *et al.*, 1996) including the constitutive cytosolic member Hsc70, the stress-induced cytosolic Hsp70, the ER-localized Grp78, and the mitochondrial Grp75 (Fink, 1999). The Hsp70 chaperones

function, along with their cochaperones the Hsp40 family members, to assist in the stabilization and folding of a broad range of substrates found in virtually all cellular compartments (Bukau and Horwich, 1998).

All Hsp70 proteins have a highly conserved amino-terminal ATPase domain that binds and hydrolyzes ATP, and a carboxy-terminal substrate binding domain. The activity of Hsp70 chaperones is regulated by Hsp40 proteins, which have a highly conserved J-domain that is required to stimulate ATP-hydrolysis, the rate-limiting step in the Hsp70 reaction cycle (Wall *et al.*, 1994, 1995; Minami *et al.*, 1996). In the ATP-bound state, the substrate binding pocket of Hsp70 is in the open conformation; hence, substrate affinity is low. Concomitant with ATP hydrolysis, Hsp40 causes a conformational switch whereby the substrate binding pocket of Hsp70 closes, facilitating the capture of nonnative protein substrates. Hsp40s, in addition to enhancing the ATPase activity of Hsp70, independently bind protein substrates, and specifically target these substrates to Hsp70, further enhancing the efficiency of the refolding cycle (Hartl, 1996). Substrate accompanies the exchange of ADP for ATP, which in the eukaryotic cytosol involves the nucleotide exchange factor Bag-1 (Alberti *et al.*, 2003). Although the presence of a highly conserved J-domain defines a protein as a member of the Hsp40 family, outside of this region the family is highly divergent. Higher eukaryotes have a large number of Hsp40 family members, and it is thought that the differential expression/localization of the various Hsp40s can impact the substrate specificity of the highly conserved Hsp70 family members (Cheetham and Caplan, 1998).

Small heat-shock proteins (sHsp) are a family of stress proteins with a molecular weight less than 40kDa. All sHsps contain a conserved, C-terminal α -crystallin domain of about 100 residues that mediates oligomeric assembly (Horwitz, 1992). In addition to the α -crystallin domain, members of this family also share a conserved WDPF motif in the amino-terminus, whereas the other regions of sHsps may be quite variable (de Jong *et al.*, 1998). Similar to Hsp90 chaperones, sHsps may transiently interact with and stabilize misfolded substrates, conceivably until Hsp70/Hsp40 system can actively refold them (Smith *et al.*, 1998).

3.2. The Ubiquitin Proteasome

When the molecular chaperone system is unable to repair misfolded proteins, targeting to the ubiquitin proteasome system can lead to selective degradation. Conjugation of ubiquitin to misfolded proteins is generally a prerequisite for proteasomal degradation and requires an enzymatic cascade that includes an E1 ubiquitin-activating enzyme, E2 ubiquitin conjugating enzyme, and an E3 ubiquitin ligase (Weissman, 2001). There is strong evidence for crosstalk between the molecular chaperone and proteasome systems. CHIP (carboxy terminus of Hsc70 interacting protein) is a protein that binds Hsc70 or Hsp70 in the mammalian cytosol and attenuates the Hsp40 stimulated ATPase and refolding activity of Hsp70 (Ballinger *et al.*, 1999). Because CHIP is an E3 ligase, it facilitates the transfer of a polyubiquitin chain to the misfolded substrate (Jiang *et al.*, 2001). In the presence of CHIP, the Hsp70 is thought to associate with BAG-1, which binds to the 26S proteasome and assists in the degradation of specific chaperone substrates (Luders *et al.*, 2000). A second cellular pathway used to degrade cellular proteins is autophagy. In general, autophagy is thought to act as a nonspecific system for the bulk removal of cellular proteins and organelles, but recent evidence (Ravikumar *et al.*, 2002; Webb *et al.*, 2003) suggests that it may play a more specific role in the degradation of aggregation-prone disease proteins. Although the aforementioned systems are normally sufficient to prevent the accumulation of misfolded proteins, it has become clear that under certain conditions, the capacity of the protein quality control system is exceeded and misfolded proteins accumulate to dangerous levels.

4. Mutations in Molecular Chaperones Responsible for Human Disease

The growing number of diseases linked to chaperone mutations demonstrates the importance of their normal activities. Mutations that disrupt the structure of and/or decrease the activity of sHsps (Clark and Muchowski, 2000) have been shown to cause several autosomal dominant genetic disorders. α -crystallin, an sHsp composed of two subunits, α A-crystallin and α B-crystallin, is a major protein constituent in the lens. A R116C mutation in α A-crystallin causes cataract, whereas a R120G mutation in α B-crystallin causes desmin-related myopathy (DRM). Although the cause of cataract formation is unclear, DRM is characterized by the accumulation of aggregates of the intermediate filament protein desmin that colocalize with α B-crystallin, suggesting a failed attempt of this sHsp to prevent the accumulation of aggregated proteins (Litt *et al.*, 1998; Vicart *et al.*, 1998; Fardeau *et al.*, 2000). Mutations in the sHsp Hsp27 (Evgrafov *et al.*, 2004) were shown recently to cause Charcot-Marie-Tooth disease and distal hereditary motor neuropathy, two diseases that are characterized by a degeneration of peripheral nerves. Four of the missense mutations occur within the α -crystallin domain of Hsp27, and one additional mutation resides in the carboxy-terminal tail. Mutations that target the α -crystallin domain of Hsp22 (Irobi *et al.*, 2004) have also been suggested to cause distal hereditary motor neuropathy.

A missense mutation (V72I) in the mitochondrial chaperonin Hsp60 (Hansen *et al.*, 2002) is responsible for an autosomal dominant form of hereditary spastic paraplegia, a neurodegenerative disorder characterized by progressive spastic weakness in the lower extremities. The assembly of chaperonin complexes that incorporate both wild-type and mutant Hsp60 subunits is thought to decrease their chaperone activity and result in haploinsufficiency. Loss of function mutations in a number of other chaperones, including the tubulin-specific chaperone E and the putative chaperonin hMKKS have also been implicated in the etiology of other heritable diseases (Barral *et al.*, 2004).

5. Protein Misfolding Diseases

There is a growing list of systemic and organ-limited diseases caused by the accumulation of extracellular or intracellular protein aggregates. In this chapter we will describe experiments focused on select neurodegenerative disorders that are intimately associated with the deposition of abnormal protein aggregates, specifically Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), and the polyglutamine expansion diseases. First, a brief overview will provide information on the genetics and pathology of each disease and discuss how universal features of protein aggregation may impart a conserved mechanism of disease pathogenesis. The following sections will outline the relevance of the molecular chaperones to each disease and review studies that characterized a role for the molecular chaperones in pathophysiology. Chaperones have been implicated in numerous protein misfolding disorders, such as the spongiform encephalopathies and oculopharyngeal muscular dystrophy, which will not be addressed in this chapter.

5.1. AD

Alzheimer's disease is the most common senile dementia, affecting ~10% of the population over the age of 65 and ~50% over the age of 80. Alzheimer's disease is characterized by widespread neuronal loss and the presence of two distinct pathologic lesions, extracellular amyloid plaques composed of the β -amyloid peptides (A β 40 and A β 42) and cytoplasmic neurofibrillary tangles composed

of the protein tau (Selkoe, 2001). Although metabolism of the amyloid precursor protein (APP) yields both A β 40 and A β 42 peptides, A β 40 is the predominant species formed in a nondisease state. In AD, the ratio of A β 42 produced relative to A β 40 increases. The A β 42 variant is more fibrillogenic than A β 40, and its accumulation is thought to accelerate disease pathogenesis. Although the overwhelming majority of AD cases are idiopathic in nature, mutations in the APP, presenilin-1 (PS1) and presenilin-2 (PS2) genes have been linked to early-onset, autosomal dominant forms of AD (FAD) (Selkoe, 2001). The APP mutation affects its proteolysis and generally increases the ratio of 42:40 of A β 42 (Goate *et al.*, 1991), whereas mutations in two presenilin genes, PS1 and PS2, which are both involved in the carboxy-terminal cleavage of A β , also enhance A β 42 accumulation suggesting that the A β 42 acts through a gain of toxic function (Levy-Lahad *et al.*, 1995; Sherrington *et al.*, 1995). Mutations in tau, a microtubule-associated protein, have not been associated with familial AD, but are linked to cases of frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), one of a growing list of disorders that are characterized by tau aggregation and deposition (Hong *et al.*, 1998; Hutton *et al.*, 1998).

5.2. PD

Parkinson's disease is the most prevalent movement disorder, affecting 1–2% of the population over age 65. Pathologically PD is defined by the progressive loss of dopaminergic neurons in the substantia nigra along with cytoplasmic aggregates that are immunopositive for the protein α -synuclein (Spillantini *et al.*, 1997). The majority of PD cases are idiopathic in nature, but rare autosomal dominant familial forms of PD are caused by mutations in the genes encoding α -synuclein, and a ubiquitin COOH-terminal hydrolase (*UCH-L1*) (Polymeropoulos *et al.*, 1997; Leroy *et al.*, 1998; Maraganore *et al.*, 1999). Autosomal recessive forms of PD are caused by mutations in the gene *Parkin*, which encodes a ubiquitin ligase (Kitada *et al.*, 1998), *DJ-1*, which encodes a protein that may act as a chaperone and/or an oxidative sensor (van Duijn *et al.*, 2001; Bonifati *et al.*, 2004), and the PTEN-induced putative kinase 1 (*PINK1*) (Valente *et al.*, 2004). PD-linked α -synuclein mutations enhance α -synuclein aggregation and are thought to act through a gain of toxic function (Caughey and Lansbury, 2003). The A53T mutation causes an increase in the kinetic of fibril accumulation, whereas A30P enhances protofibril production and delays fibril assembly, suggesting that a prefibrillar α -synuclein intermediate is a toxic species in PD (Conway *et al.*, 2000). The importance of α -synuclein expression levels in PD pathogenesis was further validated when it was discovered that a triplication of the wild-type *SNCA* gene, encoding α -synuclein, causes autosomal dominant PD (Singleton *et al.*, 2003).

5.3. ALS

Amyotrophic lateral sclerosis is a progressive paralytic disorder that results from the degeneration of motor neurons in the brainstem and spinal cord. Of the 10% of cases that are inherited (FALS), approximately 20% of these are due to autosomal dominant mutations in the gene encoding the Cu/Zn-superoxide dismutase 1 (*SOD1*) (Cleveland and Rothstein, 2001). SOD is a homodimeric metalloenzyme that catalyzes the dismutation of superoxide radical to hydrogen peroxide and molecular oxygen (Fridovich, 1986). More than 100 distinct mutations have been identified, and surprisingly, the activity of many of these mutant enzymes is unaltered (Borchelt *et al.*, 1994; Ratovitski *et al.*, 1999). In general, the SOD1 mutations decrease protein stability, increase the rate of unfolding, and increase the propensity for aggregation, suggesting that mutant SOD1 acts through a gain of toxic function (Bruijn *et al.*, 1997; Stathopoulos *et al.*, 2003). *In vitro* experiments have shown that mutant SOD1 can form both amorphous and fibrillar aggregates (Stathopoulos *et al.*, 2003). Accordingly,

inclusions containing mutant SOD are found in affected motor neurons in addition to astrocytes (Kato *et al.*, 2000).

5.4. Polyglutamine Expansion Diseases

Huntington's disease is an autosomal dominant, inherited neurodegenerative disorder caused by the expansion of a normally polymorphic CAG repeat in the *IT-15* gene, resulting in a polyglutamine expansion in the huntingtin protein (The Huntington's Disease Collaborative Research Group, 1993). Cytoplasmic and nuclear inclusion bodies containing the mutant huntingtin protein are a neuropathological hallmark of HD (DiFiglia *et al.*, 1997). A similar mutation is responsible for a growing number of diseases, including several forms of spinocerebellar ataxia (SCA), spinobulbar muscular atrophy (SBMA), and dentatorubral-pallidoluysian atrophy (DRPLA), all of which are characterized by neuronal intranuclear inclusions containing the relevant protein (Zoghbi and Orr, 2000). With the exception of SBMA, which is an X-linked disorder, polyglutamine diseases are characterized by an autosomal dominant pattern of inheritance and disease results only when the repeat expansion exceeds a critical threshold, typically 35–40 CAG repeats. The pathological threshold for disease *in vivo* correlates remarkably with the threshold for huntingtin aggregation *in vitro*, suggesting that aggregation and neurodegeneration are linked processes (Scherzinger *et al.*, 1997). *In vitro* experiments have shown that an expanded huntingtin fragment undergoes a nucleation-dependent assembly process whereby fibrillar, amyloid-like aggregates accumulate in a polyQ length-dependent manner (Wanker, 2000). It is hypothesized that the polyglutamine expansion, by enhancing the propensity of the protein to aggregate, confers a gain of toxic function to the relevant disease protein and that the specific pattern of neurodegeneration seen in each polyQ disease is the result of protein context and/or expression patterns.

6. Protein Aggregates: A Common Denominator of Neurodegenerative Disease

Remarkably, although AD, PD, ALS and HD are characterized by the aggregation of distinct proteins with unrelated primary amino acid sequences, the lesions characteristic of each disease typically contain fibrillar, amyloid-like structures with common biochemical characteristics such as detergent-insolubility, high β -sheet content, protease resistance and, the ability to bind lipophilic dyes such as Congo Red (Dobson, 2003). Whether fibrillar aggregates are pathogenic, inert, or protective is currently the subject of intense debate. Atomic force microscopy (AFM) of the aggregation of β -amyloid (A β) and α -synuclein proteins has revealed a complex assembly process involving the formation of multiple metastable intermediates, such as spherical oligomers, protofibrils, and pore-like annular structures, which may or may not be on a pathway to fibril formation (Caughey and Lansbury, 2003). It has become appreciated that prefibrillar aggregation intermediates may trigger neuronal dysfunction and initiate a cascade that culminates in neuronal death. In support of this hypothesis, the severity of dementia in AD does not correlate well with the density of insoluble amyloid plaques (Terry *et al.*, 1981; Braak and Braak, 1991; Dickson *et al.*, 1995), yet there is a robust correlation between the level of soluble A β and the magnitude of dementia, in addition to the synaptic loss (Lue *et al.*, 1999; McLean *et al.*, 1999; Wang *et al.*, 1999). Intracerebroventricular microinjection of soluble prefibrillar A β oligomers into the rat hippocampus was found to inhibit the late phase of LTP, confirming the idea that soluble A β oligomers are potent neurotoxins *in vivo* (Walsh *et al.*, 2002). These experiments must be interpreted with caution, however, because there are currently no tools available

to determine whether the proteins retain their oligomeric structure upon injection. The universality of the toxic oligomer hypothesis was recently proposed in a study demonstrating that a single monoclonal antibody was able to recognize a conformational epitope displayed by a number of disease protein intermediates, including A β , α -synuclein, and polyQ-containing peptides. Surprisingly, coincubation of the antioligomer antibody with the various oligomers blocked their toxicity when applied exogenously to cultured cells, suggesting that oligomeric intermediates of distinct disease proteins may confer toxicity through a similar mechanism (Kayed *et al.*, 2003). Although these *in vitro* results are intriguing, methodology must be developed to determine whether similar intermediates that are specific to the disease states exist *in vivo*, and if so whether the appearance of these intermediates correlates with neuronal dysfunction and/or death.

7. Molecular Chaperones: Key Regulators of Protein Aggregation and Toxicity

Postmitotic cells, such as neurons are particularly vulnerable to the effects of misfolded and/or aggregated proteins, largely as a result of the inability to dilute potentially toxic species through cell division. Moreover, neurons become increasingly vulnerable over time, as the aging process is associated with a decrease in proteasome activity and alterations in the induction and/or capacity of various molecular chaperones (Soti and Csermely, 2002). The late onset of many neurodegenerative diseases may correlate with age-dependent deficits in protein quality control, accelerated by mutations in disease-related proteins that promote the accumulation of misfolded species and accelerate the formation of insoluble deposits of aggregated protein. A common feature of AD, PD, FALS, and polyglutamine (polyQ) disease is the colocalization of fibrillar protein deposits with molecular chaperones such as Hsp70 and Hsp40 family members, components of the ubiquitin–proteasome degradation system such as ubiquitin and 20s proteasomal subunits, in addition to a large number of other cellular proteins, such as the transcription factors TBP and CBP (Sherman and Goldberg, 2001). Colocalization may reflect the irreversible sequestration of these critical cellular components in an inactive state, or in contrast, a functional, possibly beneficial interaction with the inclusion. For example, proteasomal components may become trapped within inclusions as a nonfunctional complex and by decreasing the cellular capacity for protein turnover, enhance cellular dysfunction. Conversely, the proteasome may be recruited to the inclusion as part of a defense mechanism to assist in the degradation of misfolded substrates (Sherman and Goldberg, 2001).

The techniques of fluorescence recovery after photobleaching and fluorescence loss in photobleaching have been used in a cellular model of polyglutamine disease to show that the expanded polyQ proteins, in addition to the transcription factors TBP and CBP, exhibit a low mobility within inclusions that is characteristic of their physical and possibly functional sequestration (Chai *et al.*, 2002; Kim *et al.*, 2002). Hsp70, in contrast, exhibited rapid kinetics of association and dissociation with the polyQ inclusions that were remarkably similar to the interactions observed between Hsp70 and unfolded substrates, suggesting that chaperones, rather than being sequestered, actively regulate polyQ inclusion formation. Interestingly, this unique interaction required chaperone function, as Hsp70 mutants lacking the substrate binding domain or functional ATPase activity showed markedly reduced colocalization with the inclusions (Kim *et al.*, 2002). These results suggest that the colocalization of molecular chaperones with inclusions may reflect their concentration to an area of misfolded substrates and attempts to refold, solubilize, or enhance the degradation of aggregating proteins, suppressing inclusion formation. Conversely, chaperones may promote the sequestration of prefibrillar, diffusible, potentially toxic polyglutamine assemblies into inclusions. We, and others have shown

that polyglutamine inclusion formation is a regulated cellular process that requires an intact microtubule cytoskeleton, and suggested that it may have evolved as a protective mechanism to sequester toxic, misfolded proteins, and decrease the likelihood of aberrant protein–protein interactions in the cytosol (Waelter *et al.*, 2001; Muchowski *et al.*, 2002; Taylor *et al.*, 2003).

Several genetic screens performed to identify modifiers of polyglutamine protein and α -synuclein toxicity have provided additional evidence that the molecular chaperone system plays a critical role in protein misfolding diseases (Fernandez-Funez *et al.*, 2000; Kazemi-Esfarjani and Benzer, 2000; Willingham *et al.*, 2003; Nollen *et al.*, 2004). Two screens employed a *Drosophila* model of polyglutamine disease, whereby the directed expression of either mutant ataxin-1 or an expanded polyglutamine peptide to the eye caused a degenerative phenotype characterized by retinal denegeration and a loss of normal external eye structure, including bristle arrangement and pigmentation. In both screens *Drosophila* homologs of human Hsp40 chaperones were identified as suppressors of polyglutamine-mediated neurodegeneration (Figure 8.1-1) (Fernandez-Funez *et al.*, 2000; Kazemi-Esfarjani and Benzer, 2000). We have recently performed a genome-wide screen to identify genes that enhance the toxicity of a mutant huntingtin fragment or α -synuclein (Willingham *et al.*, 2003). Consistent with the results of the *Drosophila* screens, two yeast homologs of Hsp40 (Apj1 and Hlj1) were identified in the huntingtin screen, indicating that the molecular chaperone system is critical component of the cellular defense against expanded polyglutamine proteins (Willingham *et al.*,

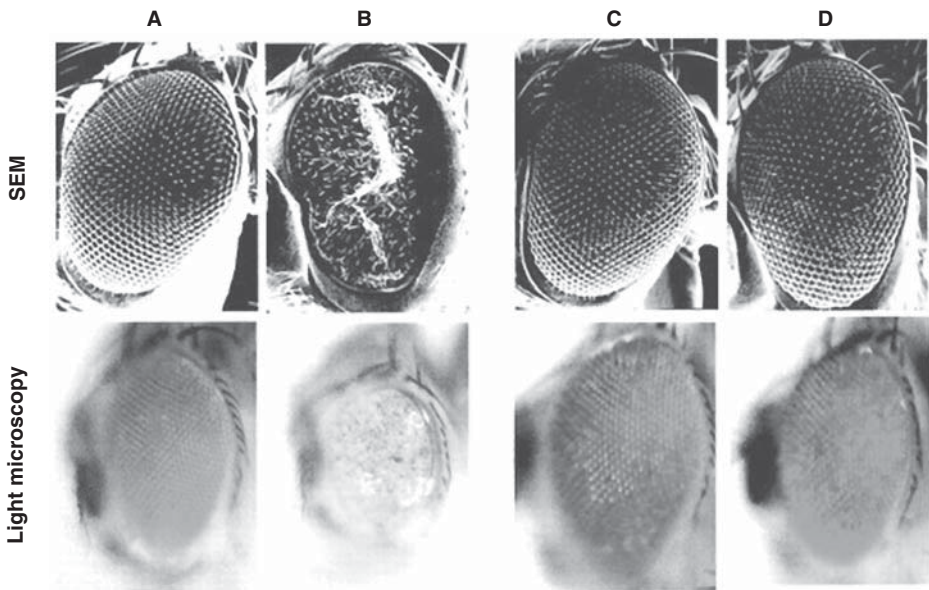


Figure 8.1-1. dHDJ1 suppresses polyQ toxicity in the fly eye. (A) Scanning electron microscopy (SEM) and light microscopy show the normal architecture of control fly eyes. (B) Expression of 127Q causes severe eye malformation. (C) The p-element insertion EU3500, which contains the sequence for dHDJ1, an Hsp40 homolog, rescues the polyQ-mediated rough eye phenotype. (D) Transgenic flies coexpressing 127Q and dHDJ1 confirm the ability of this chaperone to suppress polyQ-mediated degeneration and restore normal eye architecture. (Reprinted with permission from Kazemi-Esfarjani *et al.* Copyright 2000 AAAS.)

2003). Overall, the screen showed that the majority of gene deletions that unmasked polyglutamine toxicity were involved in the general categories of protein folding, the cellular response to stress, and ubiquitin-dependent protein catabolism. Several genes involved in the functional categories of response to stress and ubiquitin-mediated protein catabolism were also identified in the α -synuclein screen, although in contrast to the huntingtin screen, these genes were not particularly enriched, implying that the molecular chaperone system may not be the primary pathway that regulates α -synuclein toxicity, at least in a simple model system.

Rather than uncovering modifiers of aggregated protein toxicity, Nollen *et al.* (2004) performed a genome-wide RNAi feeding library screen in *Caenorhabditis elegans* to identify suppressors of polyglutamine aggregation. One hundred eighty-six genes were found to induce an early aggregation onset phenotype. The genes distributed into five functional categories including protein synthesis, protein folding, protein trafficking, and components of the proteasome. Interestingly, two members of the Hsp70 family and one DnaJ domain protein were identified as suppressors of polyQ aggregation, consistent with a specific role of the Hsp70/Hsp40 chaperone system in regulating polyglutamine aggregation (Nollen *et al.*, 2004). Collectively, the results of these screens validate the role of the molecular chaperones in regulating both the aggregation and toxicity of disease proteins. Intense research in a number of experimental systems from cell culture to mouse models has focused on relating the mechanism of chaperone action to the onset and progression of AD, PD, FALS, and polyglutamine disease (Muchowski, 2002). Below is a discussion of how manipulations of the molecular chaperone system in these models have substantiated their role in regulating the aggregation and toxicity of A β , tau, α -synuclein, SOD1, and polyglutamine proteins.

7.1. AD and Related Dementias

Immunohistochemical analysis of AD brains has shown that amyloid plaques colocalize with a number of chaperones, including α B-crystallin, Hsp27, Hsp90, ER-resident Hsp70 family member Grp78 (BiP). Although most of these chaperones are typically present as intracellular proteins, their colocalization with amyloid plaques suggests that extracellular chaperone accumulation occurs and is perhaps augmented in a disease state. Because Hsp70, in addition to several other Hsps do not contain a secretory signal sequence, extracellular release through the classical exocytic pathway is not thought to occur. Recent findings in cell culture have shown that the extracellular accumulation of chaperones may be facilitated through an interaction with lipid rafts (Broquet *et al.*, 2003). Specifically, heat shock increased the accumulation of Hsp70 in lipid rafts and correlated with a robust stimulation of Hsp70 release. Lipid raft-mediated secretion of chaperones may account for their colocalization with extracellular amyloid plaques *in vivo*.

It has been suggested that the cooperative activity of extracellular chaperones and microglial cells, the macrophages of the brain, protects neurons from the toxicity associated with extracellular A β . Activated microglia surround amyloid plaques in AD brain tissue where they may function to phagocytose and digest toxic aggregating A β peptides and plaques (Paresce *et al.*, 1996; Selkoe, 1999). *In vitro*, the addition of exogenous chaperones, such as Hsp90, Hsp70, or Grp78, along with A β peptides to microglial cultures enhanced microglial activation, concomitant with the phagocytic removal of extracellular aggregates (Kakimura *et al.*, 2001, 2002a, 2002b). The chaperones appeared to engage the TLR4 receptor, promoting the production of IL-6 and TNF- α , two pro-inflammatory cytokines that enhance the phagocytic activity of microglia (Kakimura *et al.*, 2002b). These results have been recapitulated in an *in vivo* model whereby A β 42 peptides were injected into the rat hippocampus in the presence or absence of Hsp90. Coinjection of Hsp90 and A β 42 increased the density of activated microglia surrounding the deposit, enhanced the production of IL-6 and TNF- α , and reduced total accumulation of insoluble A β 42 relative to injection of A β 42 alone, suggesting that

crosstalk between microglia and chaperones regulates plaque formation and removal *in vivo* (Takata *et al.*, 2003). What remains unclear is whether the chaperone-mediated microglial activation and subsequent phagocytosis of extracellular aggregates will delay neuronal dysfunction and/or death *in vivo*.

The effect of exogenous chaperones on the persistence and/or toxicity of A β peptides has been examined *in vitro*. Stege *et al.* (1999) showed that the addition of exogenous A β peptides to the media of cortical or hippocampal neuronal cultures caused dose-dependent toxicity, whereas coinubation of A β 40 and α B-crystallin enhanced toxicity. Transmission electron microscopy analysis of the *in vitro* aggregation of A β 40 showed that the chaperone stabilized prefibrillar A β 40 intermediates and suppressed amyloid fibril formation, suggesting that the prefibrillar A β intermediates mediated toxicity (Stege *et al.*, 1999). These results are controversial, however, because a conflicting report demonstrated that although Hsp27 and Hsp25 inhibited A β 42 fibril formation *in vitro*, α B-crystallin had no effect (Kudva *et al.*, 1997). Furthermore, an additional study suggested that α B-crystallin may actually promote fibrillization of A β 40 (Liang, 2000). These discrepancies are likely to be explained by variability in the A β starting material. The preparation of soluble, monomeric A β is technically difficult, and at the time that these studies were conducted it was not appreciated that contamination of these preparations with even a small amount of preaggregated A β can alter the kinetics of fibril accumulation.

Substantial evidence from transgenic mouse models indicates that intracellular A β accumulation may initiate cellular dysfunction prior to its accumulation in extracellular plaques (Chui *et al.*, 1999; Hsia *et al.*, 1999; Moechars *et al.*, 1999; Kumar-Singh *et al.*, 2000; Wirths *et al.*, 2001). A β / γ -secretase pathway of APP metabolism has been identified in the endoplasmic reticulum (ER) and Golgi of neurons, which results in intracellular A β 42 generation (Chyung *et al.*, 1997; Cook *et al.*, 1997; Forman *et al.*, 1997; Hartmann *et al.*, 1997; Tienari *et al.*, 1997). Mutations in the presenilin proteins, which are localized to the ER, results in increased generation of intracellular A β 42, and this property may directly reflect their association with FAD. The ER resident chaperone Grp78 was shown to bind the amyloid precursor protein (APP) and decrease the secretion of A β ₁₋₄₀ and A β ₁₋₄₂ in a HEK 293 cells transiently transfected with both APP and Grp78 (Yang *et al.*, 1998). Interestingly, levels of Grp78 were found to be dramatically reduced in cases of FAD linked to presenilin mutations, and a hypothesis has emerged suggesting that the interaction between APP and Grp78 may shield APP from interaction with β / γ -secretases and inhibit the release of toxic A β fragments (Katayama *et al.*, 1999). Overexpression of Grp78 in a more physiologically relevant *in vitro* or *in vivo* model is necessary to confirm the effect of this chaperone on APP metabolism and determine the downstream effects on neuronal survival.

Coimmunoprecipitation experiments in a transgenic *C. elegans* model expressing high levels of A β 42 in the cytoplasm has identified additional molecular chaperones, including a cytoplasmic Hsp70 and three small Hsps with homology to α B-crystallin, that interact with intracellular A β (Fonte *et al.*, 2002). Although not physiological, these results suggest, in principle, that chaperones may modulate A β aggregation/toxicity, as has been shown for other amyloid-forming proteins. The role of cytoplasmic Hsp70 in modulating the intracellular accumulation and toxicity of A β fragments has recently been examined in a doxycycline-inducible adenoviral-based system that directs intracellular A β 42 expression and accumulation into the ER of primary neuronal cultures in a regulated manner (Magrane *et al.*, 2004). Although A β 42 accumulation caused a clear induction of Hsp70, cell death occurred in a dose-dependent manner, suggesting that the levels of Hsp70 were not sufficient to mitigate A β 42 toxicity. Virally mediated overexpression of Hsp70 did, however, rescue neurons from intracellular A β 42-mediated toxicity (Magrane *et al.*, 2004). Taken together, these results suggested that the intracellular accumulation of soluble, prefibrillar forms of A β cause significant neuronal

dysfunction and eventually death, and that the molecular chaperones may alter the conformation of cytoplasmic A β in a way that either prevents the accumulation of toxic A β species, or enhances their degradation. Specifically, Hsp70 may stimulate endoplasmic reticulum-associated degradation of A β , a process whereby misfolded secretory and/or membrane proteins are recognized by a quality control mechanism in the ER, translocated into the cytosol, and subsequently degraded by the 26S proteasome.

A second defining, neuropathological feature of AD is the presence of intracellular neurofibrillary tangles (NFT) composed largely of the protein tau. Tau is normally a soluble, cytoplasmic protein that binds and stabilizes microtubules. In AD brains, tau is hyperphosphorylated, which is hypothesized to cause misfolding and subsequent release from microtubules followed by polymerization into aggregates termed paired helical filaments (PHFs) that ultimately coalesce into NFTs (Lee *et al.*, 2001). Mutations in the *MAPT* gene, which encodes tau are responsible for a related neurodegenerative disorder, Frontotemporal dementia with parkinsonism chromosome 17 type (FTDP-17), which is similar to AD, is characterized by the accumulation of aggregated tau in NFTs. The phosphorylation state of tau is a critical factor for neurodegeneration in animal models, as coexpression of tau and GSK-3 β in *Drosophila* leads to tau hyperphosphorylation, accelerated NFT formation and enhanced neurodegeneration (Jackson *et al.*, 2002). Furthermore, in mice, overexpression of a cyclin-dependent kinase-5 activator fragment, p25 caused death of cortical and hippocampal neurons that was accompanied by the accumulation of hyperphosphorylated, aggregated tau, and progressive NFT pathology (Cruz *et al.*, 2003).

Immunohistochemical analysis of AD brains showed that Hsp90 colocalizes with the periphery of NFTs, and immunoprecipitation experiments have shown that Hsp27 and Hsp70 preferentially bind to the hyperphosphorylated tau present in human brain samples (Dou *et al.*, 2003; Shimura *et al.*, 2004). Several recent studies have begun to unravel the relationship between chaperones, tau aggregation, and toxicity. Cell cultures transfected with tau constructs and treated with the antibiotic Geldanamycin (GA), which upregulates the levels of Hsp70 and Hsp90, showed a decrease in the levels of aggregated and/or phosphorylated tau and an increase in the levels of soluble tau (Dou *et al.*, 2003). Using RNAi to decrease the levels of Hsp70 and Hsp90 enhanced the accumulation of insoluble, aggregated tau, and suppressed the association of tau with microtubules, suggesting that the activity of these two chaperones serves to maintain tau in a correctly folded, functional state and promote the association of tau with microtubules (Dou *et al.*, 2003). Using a unique model in which soluble PHF tau was delivered to a human cortical neuronal cell line (HCN2A) in the presence or absence of Hsp27, it was found that delivery of Hsp27 decreased the amount of pathological, hyperphosphorylated tau, increased dephosphorylated tau, and decreased tau-mediated cell death, suggesting that Hsp27 may promote survival through the dephosphorylation of tau or assisted degradation of phosphorylated tau (Shimura *et al.*, 2004a). Interestingly, in the same study *in vitro* assays using a 26S proteasome fraction demonstrated that the presence of Hsp27 enhanced the degradation of pathological, hyperphosphorylated tau.

Crosstalk between the molecular chaperone system and the ubiquitin proteasome can facilitate the degradation of specific chaperone substrates (Murata *et al.*, 2003). CHIP, together with Hsp70 has been shown to facilitate the ubiquitination of tau in cell culture (Krichevsky and Kosik, 2002; Petrucelli *et al.*, 2004; Shimura *et al.*, 2004b). Interestingly, in both of these studies tau ubiquitination did not correspond to a decrease in tau levels, but rather increased the levels of insoluble ubiquitinated tau, suggesting that rather than facilitating degradation, CHIP-mediated ubiquitination enhanced tau aggregation. Although the study by Petrucelli *et al.* (2004) did not discriminate between the tau and phospho-tau, Shimura *et al.* (2004b) showed that CHIP, in cooperation with Hsc70 specifically ubiquitinated phosphorylated tau in COS7 cells when cotransfected with tau, CHIP, ubiquitin, and GSK-

3 β . Phospho-tau induced cell death was also rescued by CHIP expression in the same cell line. These results suggest that ubiquitination, in addition to signaling degradation, may function to partition soluble, toxic forms of phosphorylated tau into an insoluble compartment.

As of yet there have been no studies examining the effect of chaperone overexpression on tau or A β aggregation and toxicity *in vivo*. Progress in this area may have been hampered by the lack of a good *in vivo* model of tau and/or A β aggregation that recapitulates the symptoms of AD. A *C. elegans* model of tau aggregation that displays accumulation of insoluble, phosphorylated tau, and age-dependent loss of axons and neurons may prove to be an invaluable system to address these questions (Kraemer *et al.*, 2003).

7.2. PD

The Lewy Bodies characteristic of human PD colocalize with a number of molecular chaperones including Hsp70, Hsp27, Hsp40 family members Hdj-1 and Hdj-2, Hsp60, and Hsp90 in brain samples of patients diagnosed with dementia with lewy bodies (DLB) (McLean *et al.*, 2002). Dementia with lewy bodies is progressive neurodegenerative disorder that shares many of the clinical and pathological characteristics of Parkinson's disease, including α -synuclein deposition. Lewy bodies in human PD tissue colocalize with ubiquitin, and are suggested to have a composition identical to the Lewy bodies seen in DLB (Spillantini *et al.*, 1997). Transfection of neuronal cell lines or primary cultures with α -synuclein alone does not result in inclusion body formation. However, it has been established that treatment with protease inhibitors, transfection of a tagged α -synuclein, or cotransfection of a C-terminally modified α -synuclein and synphilin-1 facilitates inclusion formation *in vitro* (McLean *et al.*, 2001). Synphilin-1 was identified through a yeast two-hybrid screen as an α -synuclein interacting protein that localized to lewy bodies, yet its normal function remains unknown (Engelender *et al.*, 1999). Overexpression of Hdj1 or Hsp70 in a synuclein/synphilin-1 cell model was shown to cause a dramatic (>50%) decrease in the number of cells containing inclusions although an effect on cell viability was not reported (McLean *et al.*, 2002). In a subsequent study using an identical cell culture system, Hsp70 overexpression caused a decrease in detergent insoluble, high molecular weight α -synuclein species, and a concomitant decrease in total α -synuclein protein levels, suggesting that Hsp70 may function to enhance refolding and/or promote degradation of α -synuclein, possibly in conjunction with an E3 ligase-like CHIP (Klucken *et al.*, 2004). Furthermore, overexpression of Hsp70 caused ~20% decrease in the toxicity of transfected α -synuclein, suggesting that *in vitro*, the molecular chaperones mediate a biochemical change in the α -synuclein protein that is sufficient to diminish its toxicity (Klucken *et al.*, 2004).

The effects of altered chaperone expression on α -synuclein aggregation and/or toxicity in a genetically tractable model such as yeast or *C. elegans* have not been reported. One study in *Drosophila* has suggested that Hsp70 may play a protective role in PD (Auluck *et al.*, 2002). The GAL4/UAS system was utilized to direct expression of α -synuclein to dopaminergic neurons using the DOPA decarboxylase promoter. Expression of wild-type or mutant (A30P and A53T) α -synuclein caused dramatic (~50%) neuronal loss, and resulted in inclusion formation (Figure 8.1-2). Coexpression of human Hsp70 resulted in a complete rescue of α -synuclein-mediated toxicity, yet paradoxically had no effect on the inclusion phenotype at the level of light microscopy. The protective effect of Hsp70 may, however, result from the destabilization of toxic, prefibrillar α -synuclein intermediates that are not visible at the level of light microscopy. Specifically, chaperone activity may cause the productive refolding of α -synuclein into a less toxic state that is either degraded or sequestered in inclusions. Coexpression of a dominant negative form of *Drosophila* Hsp70 with α -synuclein accelerated dopaminergic neuron loss suggesting that endogenous chaperones modestly suppress α -synuclein-mediated neurodegeneration (Auluck *et al.*, 2002). Similar studies involving chaperone

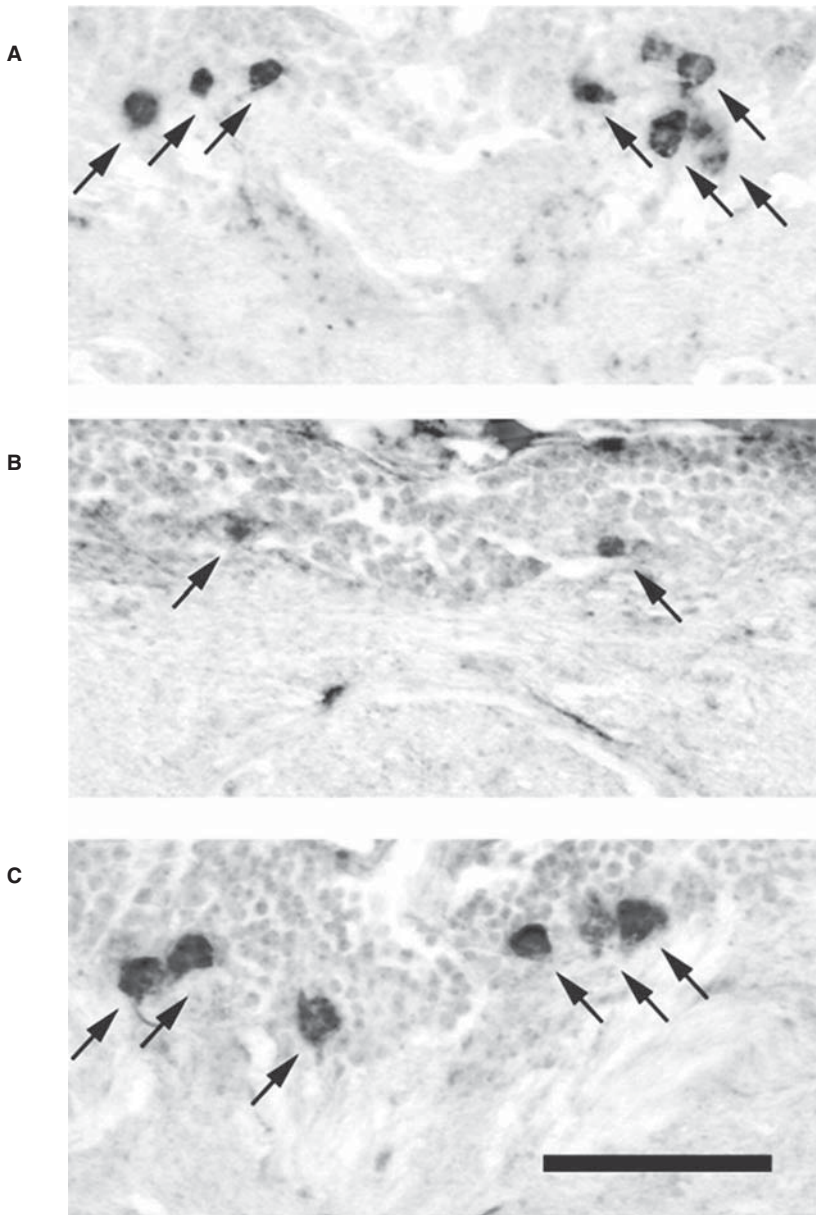


Figure 8.1-2. Hsp70 protects against α -synuclein-induced dopaminergic neuronal degeneration in *Drosophila*. Sections of fly brain were stained with an anti-TH antibody to identify dopaminergic neurons. (A) Tissue from a control fly shows many dopaminergic neurons, visible in clusters denoted with arrows. (B) Age-matched transgenic flies expressing α -synuclein show a reduction in the number of dopaminergic neurons, suggestive of α -synuclein-mediated neuronal toxicity. (C) Coexpression of α -synuclein and Hsp70 suppresses the dopaminergic neuron loss. (Reprinted with permission from Auluck *et al.* Copyright 2002 AAAS.)

overexpression have not been carried out in mouse models of PD, partially due to the lack of a model that specifically recapitulates the symptoms, neuronal loss, and pathology observed in human disease.

7.3. FALS

Although conflicting results have been obtained from human FALS tissue, in FALS transgenic mouse models mutant SOD1 aggregates colocalize with ubiquitin, proteasome components, and molecular chaperones, suggesting that protein misfolding and aberrant degradation are involved in disease pathogenesis (Watanabe *et al.*, 2001). Cellular stress, such as the accumulation of misfolded proteins, stimulates the binding and activation of heat-shock transcription factors (HSFs) to heat-shock elements (HSEs) on heat-shock promoters, increasing the synthesis of Hsps (Morimoto and Santoro, 1998). There is evidence that motor neurons have a very high threshold for induction of this response, which may be involved in the selective denervation of motor neurons in ALS. Specifically, primary spinal cord cultures failed to upregulate Hsp70 in response to heat shock, hyperthermia, glutamate excitotoxicity, or expression of mutant SOD1 (Batulan *et al.*, 2003), whereas cerebellar, cortical, and pyramidal neurons, in addition to astrocytes, upregulated Hsp70 in response to heat shock (Lowenstein *et al.*, 1991; Bechtold *et al.*, 2000; Batulan *et al.*, 2003). Expression of a constitutively active form of HSF1 caused a strong upregulation of Hsp70 in motor neurons, suggesting that a dysregulation of HSF1 may enhance the susceptibility of motor neurons to mutant SOD1. Artificially increasing the levels of Hsps would therefore be expected to protect vulnerable motor neurons from mutant SOD1 toxicity. Accordingly, intranuclear comicroinjection of expression vectors for Hsp70 and mutant SOD1 into primary motor neurons was found to reduce the toxicity of mutant SOD1, decrease SOD1 aggregation, and prolong survival (Bruening *et al.*, 1999).

A physical interaction between Hsp70 and mutant SOD1 may be required for neuroprotection, evidenced by the fact that Hsp70, in addition to Hsp40 and α B-crystallin coimmunoprecipitates with SOD1 in cell lines expressing mutant, but not wild-type SOD1 (Shinder *et al.*, 2001). Interestingly, the interaction between Hsp70 and mutant SOD was evident in total cell extracts, yet barely detected in an isolated supernatant fraction of soluble proteins, suggesting that Hsp70 interacts specifically with detergent insoluble SOD1 complexes (Shinder *et al.*, 2001). Chaperones, by influencing the solubility of mutant SOD1, may alter its compartmentalization or capacity for degradation. The fact that both Hsp70 and Hsp40 can bind mutant SOD1 suggests that their cooperative activity may significantly alter SOD1 toxicity. Overexpression of these two chaperones in the presence of mutant SOD1 in an N2a cell model results in a synergistic reduction of aggregate formation, and ameliorates toxicity to a similar extent as Hsp70 alone (Takeuchi *et al.*, 2002).

C. elegans and *Drosophila* models of FALS have not been published. A number of yeast and mouse models of FALS exist, however, the role of chaperones have not been investigated. Future studies employing overexpression or conditional knockout studies of various chaperones in these models may provide critical information on how modulation of the chaperone machinery can regulate the aggregation and toxicity of mutant SOD1 *in vivo*.

7.4. Polyglutamine Expansion Diseases

Relative to studies on A β , tau, α -synuclein and SOD1, the effect of chaperones on the aggregation and toxicity of polyglutamine proteins has been the most intensely investigated, in a diverse range of models, including *in vitro* systems, yeast, *C. elegans*, flies, and mice. Many of these studies use truncated proteins with expanded polyQ tracts, as these fragments are sufficient to cause neurodegeneration in transgenic mice and flies, as well as cell death in transfected cells (Ikeda *et al.*, 1996;

Mangiarini *et al.*, 1996; Igarashi *et al.*, 1998; Martindale *et al.*, 1998; Merry *et al.*, 1998; Warrick *et al.*, 1998). Limited evidence suggests that certain proteases, such as caspases or calpains, cleave polyglutamine-containing disease proteins, liberating toxic huntingtin fragments that may mediate toxicity *in vivo* (Goldberg *et al.*, 1996; DiFiglia *et al.*, 1997; Miyashita *et al.*, 1997; Kobayashi *et al.*, 1998; Wellington *et al.*, 1998).

In vitro experiments with an expanded huntingtin fragment (HD53Q) have shown that molecular chaperones alter the morphology and biochemical characteristics of aggregating huntingtin species. Purified Hsc70 and Hsp40 proteins, when added to aggregation reactions along with an expanded huntingtin fragment significantly suppress the formation of SDS-insoluble HD53Q aggregates (Muchowski *et al.*, 2000). Addition of these chaperones after the lag phase of aggregation had no effect, suggesting that Hsc70 and Hsp40 interact with early aggregation intermediates and alter their biochemical characteristics (Muchowski *et al.*, 2000). Similar to A β and α -synuclein, expanded huntingtin fragments form numerous prefibrillar intermediates such as spherical oligomers, annular structures, and/or amorphous aggregates prior to fibril assembly (Poirier *et al.*, 2002; Wacker *et al.*, 2004). Using AFM we have shown that Hsp70 and Hsp40 act to synergistically destabilize potentially toxic, prefibrillar HD53Q intermediates, and facilitate the accumulation of fibrillar aggregates (Figure 8.1-3; see color insert). We propose two nonmutually exclusive models to describe the mechanism of chaperone destabilization of prefibrillar polyQ intermediates. In the first model Hsp70/Hsp40 may enhance fibril accumulation as a result of the rapid assembly of chaperone-stabilized monomers into spherical intermediates, which serve as nucleation sites for fibril growth (Figure 8.1-4A). According to this model, fibrillar structures represent a lower energy state than metastable off pathway assemblies; thus, accounting for the rapid appearance of fibrils. In the second model, it is assumed that an expanded monomer forms one of several possible altered conformations, and that only one of these misfolded monomers is on the pathway for fibril formation. Hsp70/Hsp40 may facilitate the folding of a misfolded monomeric conformation of HD53Q that allows one pathway assembly to occur by monomer addition to fibril nuclei (Figure 8.1-4B). Thus, the neuroprotective effect of Hsp70/Hsp40 seen in various models of polyglutamine expansion diseases may be based on their ability to partition toxic, diffusible polyQ intermediates into relatively inert higher order aggregates.

The effect of chaperone overexpression on inclusion formation and toxicity of pathogenic polyglutamine protein/fragments has been intensely investigated in cell culture. Hdj1 overexpression consistently suppressed polyQ inclusion formation and toxicity, suggesting that these two activities are linked (Chai *et al.*, 1999; Jana *et al.*, 2000; Wyttenbach *et al.*, 2002). Although overexpression of Hsp70 decreases inclusion formation and toxicity of a truncated expanded androgen receptor, Hsp70 has been reported to decrease toxicity of an expanded huntingtin fragment in the absence of an effect on inclusion formation, suggesting that Hsp70 may facilitate protection through mechanisms that are independent of aggregation (Chai *et al.*, 1999; Jana *et al.*, 2000; Kobayashi *et al.*, 2000; Zhou *et al.*, 2001; Ishihara *et al.*, 2003). Zhou *et al.* (2001) showed that Hsp70 overexpression correlated with a decrease in caspase-3 and caspase-9 activity. Similarly, overexpression of Hsp27 had no effect on the aggregation of an expanded huntingtin fragment in COS-7 cells and increased aggregation in SKNSH cells, yet in both cell lines Hsp27 overexpression suppressed mutant huntingtin-induced cell death in a manner that correlated with a decrease in free radical production (Wyttenbach *et al.*, 2002). Cumulatively, these studies show that a reduction in toxicity does not always correspond to a visible suppression in inclusion formation, yet this does not rule out an essential role for the refolding activity of these chaperones in facilitating neuroprotection *in vivo*. Aggregation was monitored at the level of light microscopy, and the effect of chaperones on prefibrillar, presumably toxic, huntingtin assemblies could not be evaluated. Caution must be exercised in comparing various studies that rely on transient transfection due to variability in expression levels. Ultimately, the stoichiometric

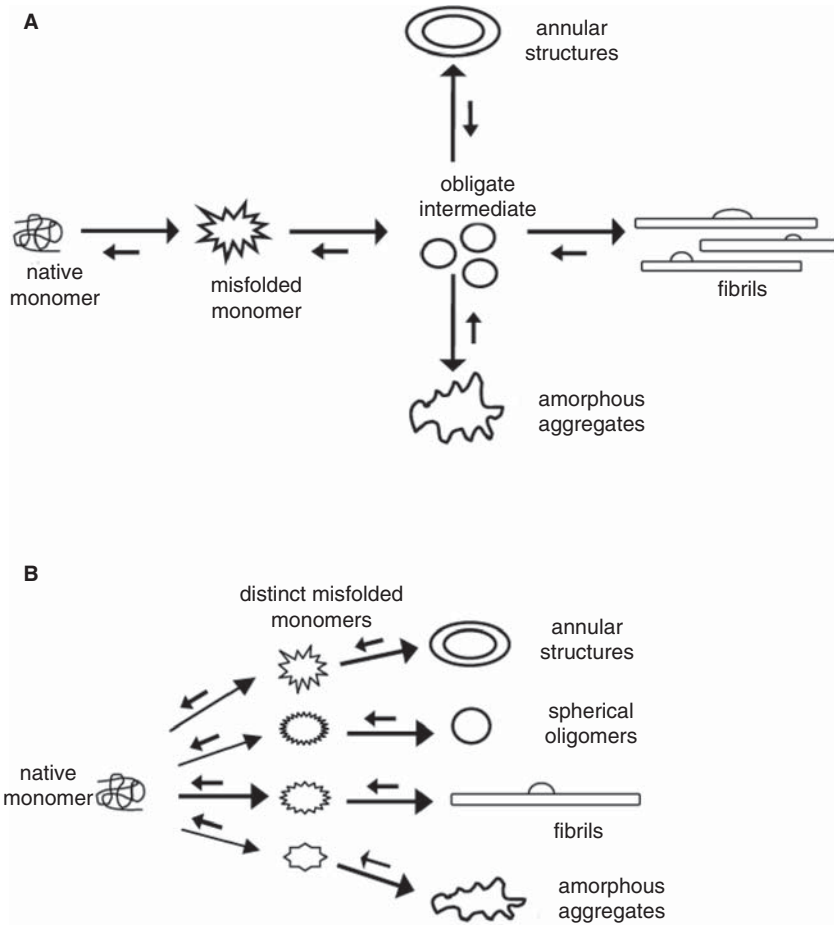


Figure 8.1-4. Two models for the assembly of polyQ proteins into amyloid-like fibrils. (A) In the first model, spherical prefibrillar structures are obligate on pathway intermediates for fibril assembly, while annular and amorphous structures are metastable off-pathway assemblies that compete with and decrease the likelihood of on-pathway interactions that promote fibril formation. Hsp70/Hsp40 may enhance the accumulation of spherical oligomers, and as a result, increase fibril assembly at the expense of competing, off-pathway aggregation reactions. (B) In the second model only one altered conformation of misfolded monomer is on-pathway for fibril formation, and thus spherical, annular, and amorphous structures are metastable off-pathway assemblies that compete with and decrease the likelihood of on pathway interactions that promote fibril formation. Hsp70/Hsp40 may stabilize a fibril-competent monomer, suppress off-pathway reactions, and as a result, enhance the accumulation of fibrillar aggregates.

levels of the chaperones relative to the polyQ proteins may determine their effect on aggregation. *In vivo* evidence increasingly suggests that a chaperone-mediated biochemical change in aggregates, such as enhanced solubility, may better correlate with neuroprotection (Chan *et al.*, 2000). Increasing the solubility of aggregating polyglutamine proteins may decrease their propensity to inhibit the proteasome, and permit their degradation through proteasomal and/or autophagic/lysosomal pathways.

Several model systems have been devised to study the effect of chaperone overexpression on polyglutamine aggregation in yeast (Krobitsch and Lindquist, 2000; Muchowski *et al.*, 2000; Meriin *et al.*, 2002). In these systems, the polyglutamine fragments typically form one to three large, perinuclear inclusion bodies. Overexpression of Ssa1 or Ydj1, conserved yeast homologs of Hsp70 and Hsp40, respectively, inhibited the formation of large, detergent-insoluble inclusions and facilitated the accumulation of numerous smaller aggregates (Muchowski *et al.*, 2000). Mutations in the yeast Hsp40 homolog Sis1, in addition to deletion of Hsp104, inhibited the seeding of huntingtin aggregates, whereas mutations in *SSA1*, *SSA2*, and *YDJ1* (an Hsp40 homolog) inhibited the expansion of huntingtin aggregates (Meriin *et al.*, 2002). Hsp70 and Hsp40, in addition to the yeast-specific Hsp104, may therefore be required for certain stages of aggregate formation. Ssa1, Ssa2, and Ydj1 mutants also displayed a decrease in polyQ-mediated toxicity (Meriin *et al.*, 2002). Consistent with these results RNAi of two Hsp70 homologs in a *C. elegans* model delayed the formation of inclusion bodies, suggesting that inclusion formation is a protective mechanism (Hsu *et al.*, 2003).

Studies in *Drosophila* have begun to provide insight into the possible mechanisms of chaperone protection of polyQ-mediated toxicity *in vivo*. When expression of a truncated form of ataxin-3 containing a 78-residue expanded polyQ tract (MJDtr-Q78) is directed to the eye, a severe degenerative phenotype results that is characterized by loss of pigment and retinal structure, in addition to the presence of intranuclear inclusions (Warrick *et al.*, 1998). Coexpression of Hsp70 results in a strong suppression of polyglutamine-induced degeneration, restoring external eye pigmentation and partially restoring retinal structure (Figure 8.1-5) (Warrick *et al.*, 1999). Expression of a dominant negative Hsp70 in the same system potentiated polyglutamine-mediated degeneration, suggesting that endogenous chaperone activity provides limited protection from the toxicity of expanded polyglutamine fragments. Strong expression of MJDtr-Q78 in all neurons normally causes lethality, but in the presence of Hsp70 male survival was increased by 2% and female survival by 30%. Most intriguingly, when inclusion formation was examined in flies overexpressing Hsp70, there were no identifiable differences in their onset, size, or number suggesting that the protective effect of Hsp70 does not require inclusion clearance (Warrick *et al.*, 1999). In a subsequent study it was found that overexpression of dHdj1 (the *Drosophila* ortholog of human Hdj1), but not dHdj2 (the fly ortholog of Hdj2) suppresses the MJDtr-Q78 degenerative eye phenotype, and that expression of both dHdj1 and Hsp70 had a synergistic effect (Chan *et al.*, 2000). Coexpressing MJDtr-Q78 with either J-domain or substrate binding domain Hdj1 mutants enhanced the degenerative phenotype, suggesting that the interaction of Hdj1 with both polyglutamine substrates and Hsp70 is critical for its protective effect. Similar to the previous study, chaperone overexpression did not coincide with a visible change in inclusion formation. Importantly, the examination of the biochemical characteristics of the inclusions formed in the presence of Hsp70 and Hsp40 showed that the chaperones enhanced the inclusion solubility, specifically increasing the appearance of soluble, monomeric protein on a Western blot of fly head homogenate (Chan *et al.*, 2000).

In contrast to the fly models, overexpression of Hsp70 in mouse models of polyglutamine disease has yielded mixed results. The R6/2 transgenic mouse model of HD expresses exon 1 of the human huntingtin gene with 150 CAG repeats and exhibits a progressive neurological phenotype with many of the characteristics of HD, including choreiform-like movements, involuntary stereotypic movements, tremor, weight loss, and neuronal inclusions (Mangiarini *et al.*, 1997). With the exception of a minor decrease in weight loss, overexpression of Hsp70 in these mice to about 5–15 times normal

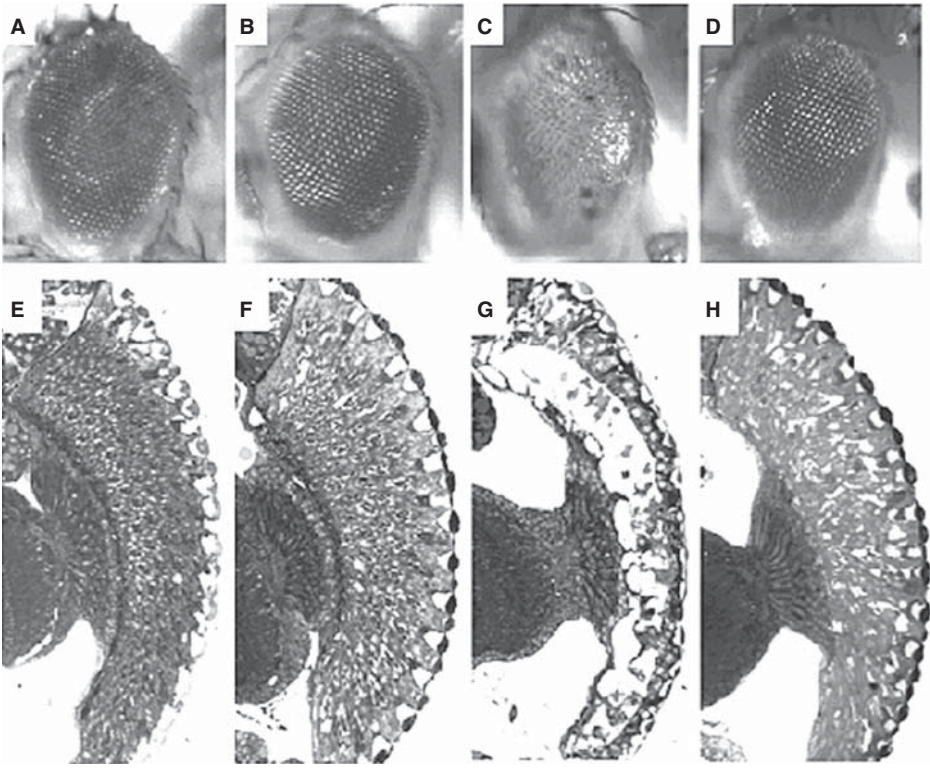


Figure 8.1-5. Hsp70 suppresses polyglutamine-induced neurodegeneration in *Drosophila*. (A–D) The external features of fly eyes, whereas (E–F) show retinal sections. (A and E) Control flies have normal eye architecture. (B and F) Overexpression of Hsp70 has no effect on external or retinal morphology. (C and G) Flies expressing an expanded polyglutamine protein (MJDtr-Q78) show external and retinal degeneration. (D and H) Co-overexpression of Hsp70 with MJDtr-Q78 ameliorates polyQ-dependent degeneration. (Reprinted with permission from Warrick *et al.* Copyright 1999 Nature America Inc.)

levels had almost no effect on the R6/2 behavioral or neuropathological phenotype (Hansson *et al.*, 2003). As suggested by the fly studies, cooverexpression of Hsp70 and Hsp40 may prove critical to detect a neuroprotective effect in this model. Overexpression of Hsp70 alone has, however, been successful in other mouse models of polyQ disease. Crossing the B05 line of SCA1 transgenic mice, which expresses ataxin-1 82Q at 50 to 100 times endogenous levels, to an Hsp70 transgenic line that has a 10-fold increase in Hsp70 expression levels resulted in a significant improvement of the behavioral and neuropathological phenotypes (Cummings *et al.*, 2001). Similar to the fly models, protection occurred in the absence of a visible change in inclusion morphology. Due to technical difficulties associated with ataxin-1 extraction, alterations in the biochemical characteristics of the inclusions were not reported. There was, however, a dose-dependent effect of Hsp70 on the motor phenotype, because mice homozygous for Hsp70 showed a more robust improvement than hemizygous mice. The

B05/Hsp70(tg/tg) mice also showed a dramatic improvement in Purkinje cell morphology, including improved dendritic arborization and less disruption of the Purkinje cell layer (**Figure 8.1-6; see color insert**). Similar to the SCA1 model, overexpression of Hsp70 in a mouse model of SBMA caused a dose-dependent improvement in motor coordination (Adachi *et al.*, 2003). Immunohistochemistry revealed a decrease in the nuclear localization of the mutant AR, which correlated with a decrease in the high molecular weight and monomeric protein in a Western blot, suggesting that Hsp70 overexpression enhanced degradation the mutant protein.

8. Chaperones as a Potential Drug Target

8.1. Chemical Chaperones

It has become clear that the development of chaperone mimetics or molecules that upregulate chaperone expression may be of benefit for a number of neurodegenerative disorders. Caution must be exercised, however, as drastic upregulation of chaperones may lead to undesirable side effects, such as cancer. A delicate balance of the various of chaperones is likely to be required for a beneficial, neuroprotective effect. Several low molecular weight compounds, such as the organic solvent dimethyl sulfoxide (DMSO) in addition to the cellular osmolytes glycerol, trimethylamine N-oxide (TMAO), and trehalose increase the stability of native proteins *in vitro* (Hottiger *et al.*, 1994; Tatzelt *et al.*, 1996; Wang and Bolen, 1997). Treatment of cells expressing a truncated, expanded ataxin-3 protein with either DMSO, glycerol, or TMAO suppressed aggregate formation and cell death, highlighting the potential therapeutic value of chemical chaperones (Yoshida *et al.*, 2002). Similar to the Hsps, the synthesis of trehalose in yeast is upregulated in response to cellular stress (Attfield, 1987), where it serves a dual role to enhance protein stability and maintain aggregation-prone proteins in a partially folded state that facilitates subsequent refolding by the molecular chaperones (Singer and Lindquist, 1998). Trehalose was identified in an *in vitro* screen for inhibitors of polyglutamine aggregation, and its efficacy was subsequently evaluated in the R6/2 transgenic model of Huntington's disease (Tanaka *et al.*, 2004). Although the effects observed were rather mild, administration of 2% trehalose in the drinking water reduced brain atrophy, improved motor dysfunction, and extended the life span of transgenic mice.

8.2. Drugs That Upregulate Chaperone Expression

Chemical chaperones, in combination with a pharmacological agent that upregulates the synthesis of molecular chaperones, may prove to be a valid therapeutic approach to protein misfolding diseases. The exposure of stressed cells to nonnative protein is a key proximal inducer of HSF1 activation, which leads to an increase in the expression of a number of Hsps (Ananthan *et al.*, 1986). Hsp90 is a part of a complex that negatively regulates the activity of HSF1 (Zou *et al.*, 1998), and is thus an attractive therapeutic target. Geldanamycin is a naturally occurring benzoquinone ansamycin that binds to the ATP binding site of Hsp90 and interrupts its interaction with HSF1, promoting HSF1 activation (Prodromou *et al.*, 1997; Zou *et al.*, 1998). Geldanamycin has been shown to inhibit huntingtin aggregation in a cell culture model of Huntington's disease and protect against α -synuclein toxicity in flies, despite the persistence of Lewy body-like inclusions (Sittler *et al.*, 2001; Auluck and Bonini, 2002). In the future, the investigation of chaperone inducing compounds such as the macrocyclic antifungal antibiotic radicicol, which has been found to act through the same mechanism as GA, yet has a 50-fold greater affinity for Hsp90 (Roe *et al.*, 1999), or the hydroxylamine derivative

bimoclolmol that binds to HSF-1 and causes an increase in the duration of its binding to HSEs (Hargitai *et al.*, 2003) may yield similar results, and foster the development of new therapeutic strategies for protein misfolding diseases.

9. Abbreviations

A β	Amyloid β peptide
AD	Alzheimer's disease
AFM	Atomic force microscopy
ALS	Amyotropic lateral sclerosis
APP	Amyloid precursor protein
CHIP	Carboxy terminus of Hsc70 interacting protein
DLB	Dementia with Lewy bodies
DMSO	Dimethyl sulfoxide
DRPLA	Dentatorubral-pallidoluysian atrophy
ER	Endoplasmic reticulum
HSE	Heat-shock element
HSF	Heat-shock transcription factor
Hsp	Heat-shock proteins
FAD	Autosomal dominant forms of AD
FALS	Inherited amyotropic lateral sclerosis
GA	Geldanamycin
HD	Huntington's disease
MJDtr-Q78	Ataxin-3 containing a 78-residue expanded polyQ tract
NFT	Neurofibrillary tangles
PD	Parkinson's disease
PHF	Paired helical filament
PS1	Presenilin-1
PS2	Presenilin-2
SCA	Spinocerebellar ataxia
SBMA	Spinobulbar muscular atrophy
SOD1	Cu/Zn-superoxide dismutase 1
TMAO	Trimethylamine N-oxide

References

- Adachi, H., Katsuno, M., Minamiyama, M., Sang, C., Pagoulatos, G., Angelidis, C., Kusakabe, M., Yoshiki, A., Kobayashi, Y., Doyu, M., and Sobue, G. (2003). Heat shock protein 70 chaperone overexpression ameliorates phenotypes of the spinal and bulbar muscular atrophy transgenic mouse model by reducing nuclear-localized mutant androgen receptor protein. *J. Neurosci.* 23:2203–2211.
- Alberti, S., Esser, C., and Hohfeld, J. (2003). BAG-1—a nucleotide exchange factor of Hsc70 with multiple cellular functions. *Cell Stress Chaperones* 8:225–231.
- Ananthan, J., Goldberg, A.L., and Voellmy, R. (1986). Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Science* 232:522–524.
- Anfinsen, C.B. (1973). Principles that govern the folding of protein chains. *Science* 181:223–230.
- Attfield, P.V. (1987). Trehalose accumulates in *Saccharomyces cerevisiae* during exposure to agents that induce heat shock response. *FEBS Lett.* 225:259–263.

- Auluck, P.K., and Bonini, N.M. (2002). Pharmacological prevention of Parkinson disease in *Drosophila*. *Nat. Med.* 8:1185–1186.
- Auluck, P.K., Chan, H.Y., Trojanowski, J.Q., Lee, V.M., and Bonini, N.M. (2002). Chaperone suppression of alpha-synuclein toxicity in a *Drosophila* model for Parkinson's disease. *Science* 295:865–868.
- Ballinger, C.A., Connell, P., Wu, Y., Hu, Z., Thompson, L.J., Yin, L.Y., and Patterson, C. (1999). Identification of CHIP, a novel tetratricopeptide repeat-containing protein that interacts with heat shock proteins and negatively regulates chaperone functions. *Mol. Cell. Biol.* 19:4535–4545.
- Barral, J.M., Broadley, S.A., Schaffar, G., and Hartl, F.U. (2004). Roles of molecular chaperones in protein misfolding diseases. *Semin. Cell Dev. Biol.* 15:17–29.
- Batulan, Z., Shinder, G.A., Minotti, S., He, B.P., Doroudchi, M.M., Nalbantoglu, J., Strong, M.J., and Durham, H.D. (2003). High threshold for induction of the stress response in motor neurons is associated with failure to activate HSF1. *J. Neurosci.* 23:5789–5798.
- Bechtold, D.A., Rush, S.J., and Brown, I.R. (2000). Localization of the heat-shock protein Hsp70 to the synapse following hyperthermic stress in the brain. *J. Neurochem.* 74:641–646.
- Bonifati, V., Oostra, B.A., and Heutink, P. (2004). Linking DJ-1 to neurodegeneration offers novel insights for understanding the pathogenesis of Parkinson's disease. *J. Mol. Med.* 82:163–174.
- Borchelt, D.R., Lee, M.K., Slunt, H.S., Guarnieri, M., Xu, Z.S., Wong, P.C., Brown, R.H., Jr., Price, D.L., Sisodia, S.S., and Cleveland, D.W. (1994). Superoxide dismutase 1 with mutations linked to familial amyotrophic lateral sclerosis possesses significant activity. *Proc. Natl. Acad. Sci. USA* 91:8292–8296.
- Braak, H., and Braak, E. (1991). Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol. (Berl.)* 82:239–259.
- Broquet, A.H., Thomas, G., Masliah, J., Trugnan, G., and Bachelet, M. (2003). Expression of the molecular chaperone Hsp70 in detergent-resistant microdomains correlates with its membrane delivery and release. *J. Biol. Chem.* 278:21601–21606.
- Bruening, W., Roy, J., Giasson, B., Figlewicz, D.A., Mushynski, W.E., and Durham, H.D. (1999). Up-regulation of protein chaperones preserves viability of cells expressing toxic Cu/Zn-superoxide dismutase mutants associated with amyotrophic lateral sclerosis. *J. Neurochem.* 72:693–699.
- Brujin, L.I., Becher, M.W., Lee, M.K., Anderson, K.L., Jenkins, N.A., Copeland, N.G., Sisodia, S.S., Rothstein, J.D., Borchelt, D.R., Price, D.L., and Cleveland, D.W. (1997). ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. *Neuron* 18:327–338.
- Bukau, B., and Horwich, A.L. (1998). The Hsp70 and Hsp60 chaperone machines. *Cell* 92:351–366.
- Caughey, B., and Lansbury, P.T. (2003). Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders. *Annu. Rev. Neurosci.* 26:267–298.
- Chai, Y., Koppenhafer, S.L., Bonini, N.M., and Paulson, H.L. (1999). Analysis of the role of heat shock protein (Hsp) molecular chaperones in polyglutamine disease. *J. Neurosci.* 19:10338–10347.
- Chai, Y., Shao, J., Miller, V.M., Williams, A., and Paulson, H.L. (2002). Live-cell imaging reveals divergent intracellular dynamics of polyglutamine disease proteins and supports a sequestration model of pathogenesis. *Proc. Natl. Acad. Sci. USA* 99:9310–9315.
- Chan, H.Y., Warrick, J.M., Gray-Board, G.L., Paulson, H.L., and Bonini, N.M. (2000). Mechanisms of chaperone suppression of polyglutamine disease: selectivity, synergy and modulation of protein solubility in *Drosophila*. *Hum. Mol. Genet.* 9:2811–2820.
- Cheetham, M.E., and Caplan, A.J. (1998). Structure, function and evolution of DnaJ: conservation and adaptation of chaperone function. *Cell Stress Chaperones* 3:28–36.
- Chui, D.H., Tanahashi, H., Ozawa, K., Ikeda, S., Checler, F., Ueda, O., Suzuki, H., Araki, W., Inoue, H., Shirofani, K., Takahashi, K., Gallyas, F., and Tabira, T. (1999). Transgenic mice with Alzheimer presenilin 1 mutations show accelerated neurodegeneration without amyloid plaque formation. *Nat. Med.* 5:560–564.
- Chyng, A.S., Greenberg, B.D., Cook, D.G., Doms, R.W., and Lee, V.M. (1997). Novel beta-secretase cleavage of beta-amyloid precursor protein in the endoplasmic reticulum/intermediate compartment of NT2N cells. *J. Cell Biol.* 138:671–680.
- Citri, A., Kochupurakkal, B.S., and Yarden, Y. (2004). The achilles heel of ErbB-2/HER2: regulation by the Hsp90 chaperone machine and potential for pharmacological intervention. *Cell Cycle* 3:51–60.
- Clark, J.I., and Muchowski, P.J. (2000). Small heat-shock proteins and their potential role in human disease. *Curr. Opin. Struct. Biol.* 10:52–59.
- Cleveland, D.W., and Rothstein, J.D. (2001). From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. *Nat. Rev. Neurosci.* 2:806–819.

- Conway, K.A., Lee, S.J., Rochet, J.C., Ding, T.T., Harper, J.D., Williamson, R.E., and Lansbury, P.T., Jr. (2000). Accelerated oligomerization by Parkinson's disease linked alpha-synuclein mutants. *Ann. N. Y. Acad. Sci.* 920:42–45.
- Cook, D.G., Forman, M.S., Sung, J.C., Leight, S., Kolson, D.L., Iwatsubo, T., Lee, V.M., and Doms, R.W. (1997). Alzheimer's A beta (1–42) is generated in the endoplasmic reticulum/intermediate compartment of NT2N cells. *Nat. Med.* 3:1021–1023.
- Cruz, J.C., Tseng, H.C., Goldman, J.A., Shih, H., and Tsai, L.H., (2003). Aberrant Cdk5 activation by p25 triggers pathological events leading to neurodegeneration and neurofibrillary tangles. *Neuron* 40:471–483.
- Cummings, C.J., Sun, Y., Opal, P., Antalffy, B., Mestrl, R., Orr, H.T., Dillmann, W.H., and Zoghbi, H.Y., (2001). Overexpression of inducible HSP70 chaperone suppresses neuropathology and improves motor function in SCA1 mice. *Hum. Mol. Genet.* 10:1511–1518.
- de Jong, W.W., Caspers, G.J., and Leunissen, J.A. (1998). Genealogy of the alpha-crystallin—small heat-shock protein superfamily. *Int. J. Biol. Macromol.* 22:151–162.
- Dickson, D.W., Crystal, H.A., Bevana, C., Honer, W., Vincent, I., and Davies, P. (1995). Correlations of synaptic and pathological markers with cognition of the elderly. *Neurobiol. Aging* 16:285–298; discussion 298–304.
- DiFiglia, M., Sapp, E., Chase, K.O., Davies, S.W., Bates, G.P., Vonsattel, J.P., and Aronin, N. (1997). Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science* 277:1990–1993.
- Dobson, C.M. (2003). Protein folding and misfolding. *Nature* 426:884–890.
- Dou, F., Netzer, W.J., Tanemura, K., Li, F., Hartl, F.U., Takashima, A., Gouras, G.K., Greengard, P., and Xu, H. (2003). Chaperones increase association of tau protein with microtubules. *Proc. Natl. Acad. Sci. USA* 100:721–726.
- Ellis, R.J. (2001). Macromolecular crowding: an important but neglected aspect of the intracellular environment. *Curr. Opin. Struct. Biol.* 11:114–119.
- Ellis, R.J., and Hartl, F.U. (1999). Principles of protein folding in the cellular environment. *Curr. Opin. Struct. Biol.* 9:102–110.
- Engelender, S., Kaminsky, Z., Guo, X., Sharp, A.H., Amaravi, R.K., Kleiderlein, J.J., Margolis, R.L., Troncoso, J.C., Lanahan, A.A., Worley, P.F., Dawson, V.L., Dawson, T.M., and Ross, C.A. (1999). Synphilin-1 associates with alpha-synuclein and promotes the formation of cytosolic inclusions. *Nat. Genet.* 22:110–114.
- Evgrafov, O.V., Mersyanova, I., Irobi, J., Van Den Bosch, L., Dierick, I., Leung, C.L., Schagina, O., Verpoorten, N., Van Impe, K., Fedotov, V., Dadali, E., Auer-Grumbach, M., Windpassinger, C., Wagner, K., Mitrovic, Z., Hilton-Jones, D., Talbot, K., Martin, J.J., Vasserman, N., Tverskaya, S., Polyakov, A., Liem, R.K., Gettemans, J., Robberecht, W., De Jonghe, P., and Timmerman, V. (2004). Mutant small heat-shock protein 27 causes axonal Charcot-Marie-Tooth disease and distal hereditary motor neuropathy. *Nat. Genet.* 36:602–606.
- Fardeau, M., Vicart, P., Caron, A., Chateau, D., Chevallay, M., Collin, H., Chapon, F., Duboc, D., Eymard, B., Tome, F.M., Dupret, J.M., Paulin, D., and Guicheney, P. (2000). Familial myopathy with desmin storage seen as a granulo-filamentar, electron-dense material with mutation of the alphaB-crystallin gene. *Rev. Neurol. (Paris)* 156: 497–504.
- Fernandez-Funez, P., Nino-Rosales, M.L., de Gouyon, B., She, W.C., Luchak, J.M., Martinez, P., Turiegano, E., Benito, J., Capovilla, M., Skinner, P.J., McCall, A., Canal, I., Orr, H.T., Zoghbi, H.Y., and Botas, J. (2000). Identification of genes that modify ataxin-1-induced neurodegeneration. *Nature* 408:101–106.
- Fink, A.L. (1999). Chaperone-mediated protein folding. *Physiol. Rev.* 79:425–449.
- Fonte, V., Kapulkin, V., Taft, A., Fluet, A., Friedman, D., and Link, C.D. (2002). Interaction of intracellular beta amyloid peptide with chaperone proteins. *Proc. Natl. Acad. Sci. USA* 99:9439–9444.
- Forman, M.S., Cook, D.G., Leight, S., Doms, R.W., and Lee, V.M. (1997). Differential effects of the swedish mutant amyloid precursor protein on beta-amyloid accumulation and secretion in neurons and nonneuronal cells. *J. Biol. Chem.* 272:32247–32253.
- Fridovich, I. (1986). Superoxide dismutases. *Adv. Enzymol. Relat. Areas Mol. Biol.* 58:61–97.
- Glover, J.R., and Lindquist, S. (1998). Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. *Cell* 94:73–82.
- Goate, A., Chartier-Harlin, M.C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., et al. (1991). Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* 349:704–706.
- Goldberg, Y.P., Nicholson, D.W., Rasper, D.M., Kalchman, M.A., Koide, H.B., Graham, R.K., Bromm, M., Kazemi-Esfarjani, P., Thornberry, N.A., Vaillancourt, J.P., and Hayden, M.R. (1996). Cleavage of huntingtin by apopain, a proapoptotic cysteine protease, is modulated by the polyglutamine tract. *Nat. Genet.* 13:442–449.
- Hansen, J.J., Durr, A., Courru-Rebeix, I., Georgopoulos, C., Ang, D., Nielsen, M.N., Davoine, C.S., Brice, A., Fontaine, B., Gregersen, N., and Bross, P. (2002). Hereditary spastic paraplegia SPG13 is associated with a mutation in the gene encoding the mitochondrial chaperonin Hsp60. *Am. J. Hum. Genet.* 70:1328–1332.

- Hansson, O., Nylandsted, J., Castilho, R.F., Leist, M., Jaattela, M., and Brundin, P. (2003). Overexpression of heat shock protein 70 in R6/2 Huntington's disease mice has only modest effects on disease progression. *Brain Res.* 970: 47–57.
- Hargitai, J., Lewis, H., Boros, I., Racz, T., Fiser, A., Kurucz, I., Benjamin, I., Vigh, L., Penzes, Z., Csermely, P., and Latchman, D.S. (2003). Bimocinolol, a heat shock protein co-inducer, acts by the prolonged activation of heat shock factor-1. *Biochem. Biophys. Res. Commun.* 307:689–695.
- Hartl, F.U. (1996). Molecular chaperones in cellular protein folding. *Nature* 381:571–579.
- Hartl, F.U., and Hayer-Hartl, M. (2002). Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* 295:1852–1858.
- Hartmann, T., Bieger, S.C., Bruhl, B., Tienari, P.J., Ida, N., Allsop, D., Roberts, G.W., Masters, C.L., Dotti, C.G., Unsicker, K., and Beyreuther, K. (1997). Distinct sites of intracellular production for Alzheimer's disease A beta40/42 amyloid peptides. *Nat. Med.* 3:1016–1020.
- Hong, M., Zhukareva, V., Vogelsberg-Ragaglia, V., Wszolek, Z., Reed, L., Miller, B.I., Geschwind, D.H., Bird, T.D., McKeel, D., Goate, A., Morris, J.C., Wilhelmsen, K.C., Schellenberg, G.D., Trojanowski, J.Q., and Lee, V.M. (1998). Mutation-specific functional impairments in distinct tau isoforms of hereditary FTDP-17. *Science* 282:1914–1917.
- Horwitz, J. (1992). Alpha-crystallin can function as a molecular chaperone. *Proc. Natl. Acad. Sci. USA* 89: 10449–10453.
- Hottiger, T., De Virgilio, C., Hall, M.N., Boller, T., and Wiemken, A. (1994). The role of trehalose synthesis for the acquisition of thermotolerance in yeast. II. Physiological concentrations of trehalose increase the thermal stability of proteins in vitro. *Eur. J. Biochem.* 219:187–193.
- Hsia, A.Y., Masliah, E., McConlogue, L., Yu, G.Q., Tatsuno, G., Hu, K., Kholodenko, D., Malenka, R.C., Nicoll, R.A., and Mucke, L. (1999). Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. *Proc. Natl. Acad. Sci. USA* 96:3228–3233.
- Hsu, A.L., Murphy, C.T., and Kenyon, C. (2003). Regulation of aging and age-related disease by DAF-16 and heat-shock factor. *Science* 300:1142–1145.
- Hutton, M., Lendon, C. L., Rizzu, P., Baker, M., Froelich, S., Houlden, H., Pickering-Brown, S., Chakraverty, S., Isaacs, A., Grover, A., Hackett, J., Adamson, J., Lincoln, S., Dickson, D., Davies, P., Petersen, R.C., Stevens, M., de Graaff, E., Wauters, E., van Baren, J., Hillebrand, M., Joosse, M., Kwon, J.M., Nowotny, P., Heutink, P., and *et al.* (1998). Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. *Nature* 393: 702–705.
- Igarashi, S., Koide, R., Shimohata, T., Yamada, M., Hayashi, Y., Takano, H., Date, H., Oyake, M., Sato, T., Sato, A., Egawa, S., Ikeuchi, T., Tanaka, H., Nakano, R., Tanaka, K., Hozumi, I., Inuzuka, T., Takahashi, H., and Tsuji, S. (1998). Suppression of aggregate formation and apoptosis by transglutaminase inhibitors in cells expressing truncated DRPLA protein with an expanded polyglutamine stretch. *Nat. Genet.* 18:111–117.
- Ikeda, H., Yamaguchi, M., Sugai, S., Aze, Y., Narumiya, S., and Kakizuka, A. (1996). Expanded polyglutamine in the Machado-Joseph disease protein induces cell death in vitro and in vivo. *Nat. Genet.* 13:196–202.
- Irobi, J., Van Impe, K., Seeman, P., Jordanova, A., Dierick, I., Verpoorten, N., Michalik, A., De Vriendt, E., Jacobs, A., Van Gerwen, V., Vennekens, K., Mazanec, R., Tournev, I., Hilton-Jones, D., Talbot, K., Kremensky, I., Van Den Bosch, L., Robberecht, W., Van Vandeckerckhove, J., Broeckhoven, C., Gettemans, J., De Jonghe, P., and Timmerman, V. (2004). Hot-spot residue in small heat-shock protein 22 causes distal motor neuropathy. *Nat. Genet.* 36: 597–601.
- Ishihara, K., Yamagishi, N., Saito, Y., Adachi, H., Kobayashi, Y., Sobue, G., Ohtsuka, K., and Hatayama, T. (2003). Hsp105alpha suppresses the aggregation of truncated androgen receptor with expanded CAG repeats and cell toxicity. *J. Biol. Chem.* 278:25143–25150.
- Jackson, G.R., Wiedau-Pazos, M., Sang, T.K., Wagle, N., Brown, C.A., Massachi, S., and Geschwind, D.H. (2002). Human wild-type tau interacts with wingless pathway components and produces neurofibrillary pathology in *Drosophila*. *Neuron* 34:509–519.
- Jana, N.R., Tanaka, M., Wang, G., and Nukina, N. (2000). Polyglutamine length-dependent interaction of Hsp40 and Hsp70 family chaperones with truncated N-terminal huntingtin: their role in suppression of aggregation and cellular toxicity. *Hum. Mol. Genet.* 9:2009–2018.
- Jiang, J., Ballinger, C.A., Wu, Y., Dai, Q., Cyr, D.M., Hohfeld, J., and Patterson, C. (2001). CHIP is a U-box-dependent E3 ubiquitin ligase: identification of Hsc70 as a target for ubiquitylation. *J. Biol. Chem.* 276:42938–42944.
- Kakimura, J., Kitamura, Y., Taniguchi, T., Shimohama, S., and Gebicke-Haerter, P.J. (2001). Bip/GRP78-induced production of cytokines and uptake of amyloid-beta(1–42) peptide in microglia. *Biochem. Biophys. Res. Commun.* 281: 6–10.

- Kakimura, J., Kitamura, Y., Takata, K., Tsuchiya, D., Taniguchi, T., Gebicke-Haerter, P.J., Smith, M.A., Perry, G., and Shimohama, S. (2002a). Possible involvement of ER chaperone Grp78 on reduced formation of amyloid-beta deposits. *Ann. N. Y. Acad. Sci.* 977:327–332.
- Kakimura, J., Kitamura, Y., Takata, K., Umeki, M., Suzuki, S., Shibagaki, K., Taniguchi, T., Nomura, Y., Gebicke-Haerter, P.J., Smith, M.A., Perry, G., and Shimohama, S. (2002b). Microglial activation and amyloid-beta clearance induced by exogenous heat-shock proteins. *FASEB J.* 16:601–603.
- Katayama, T., Imaizumi, K., Sato, N., Miyoshi, K., Kudo, T., Hitomi, J., Morihara, T., Yoneda, T., Gomi, F., Mori, Y., Nakano, Y., Takeda, J., Tsuda, T., Itoyama, Y., Murayama, O., Takashima, A., St. George-Hyslop, P., Takeda, M., and Tohyama, M. (1999). Presenilin-1 mutations downregulate the signalling pathway of the unfolded-protein response. *Nat. Cell Biol.* 1:479–485.
- Kato, S., Takikawa, M., Nakashima, K., Hirano, A., Cleveland, D.W., Kusaka, H., Shibata, N., Kato, M., Nakano, I., and Ohama, E. (2000). New consensus research on neuropathological aspects of familial amyotrophic lateral sclerosis with superoxide dismutase 1 (SOD1) gene mutations: inclusions containing SOD1 in neurons and astrocytes. *Amyotroph. Lateral Scler. Other Motor Neuron Disord.* 1:163–184.
- Kayed, R., Head, E., Thompson, J.L., McIntire, T.M., Milton, S.C., Cotman, C.W., and Glabe, C.G. (2003). Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science* 300:486–489.
- Kazemi-Esfarjani, P., and Benzer, S. (2000). Genetic suppression of polyglutamine toxicity in *Drosophila*. *Science* 287:1837–1840.
- Kim, S., Nollen, E.A., Kitagawa, K., Bindokas, V.P., and Morimoto, R.I. (2002). Polyglutamine protein aggregates are dynamic. *Nat. Cell Biol.* 4:826–831.
- Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 392: 605–608.
- Klucken, J., Shin, Y., Masliah, E., Hyman, B.T., and McLean, P.J. (2004). Hsp70 reduces alpha-synuclein aggregation and toxicity. *J. Biol. Chem.*
- Kobayashi, Y., Miwa, S., Merry, D.E., Kume, A., Mei, L., Doyu, M., and Sobue, G. (1998). Caspase-3 cleaves the expanded androgen receptor protein of spinal and bulbar muscular atrophy in a polyglutamine repeat length-dependent manner. *Biochem. Biophys. Res. Commun.* 252:145–150.
- Kobayashi, Y., Kume, A., Li, M., Doyu, M., Hata, M., Ohtsuka, K., and Sobue, G. (2000). Chaperones Hsp70 and Hsp40 suppress aggregate formation and apoptosis in cultured neuronal cells expressing truncated androgen receptor protein with expanded polyglutamine tract. *J. Biol. Chem.* 275:8772–8778.
- Kraemer, B.C., Zhang, B., Leverenz, J.B., Thomas, J.H., Trojanowski, J.Q., and Schellenberg, G.D. (2003). Neurodegeneration and defective neurotransmission in a *Caenorhabditis elegans* model of tauopathy. *Proc. Natl. Acad. Sci. USA* 100:9980–9985.
- Krichevsky, A.M., and Kosik, K.S. (2002). RNAi functions in cultured mammalian neurons. *Proc. Natl. Acad. Sci. USA* 99:11926–11929.
- Krobitsch, S., and Lindquist, S. (2000). Aggregation of huntingtin in yeast varies with the length of the polyglutamine expansion and the expression of chaperone proteins. *Proc. Natl. Acad. Sci. USA* 97:1589–1594.
- Kudva, Y.C., Hiddinga, H.J., Butler, P.C., Mueske, C.S., and Eberhardt, N.L. (1997). Small heat shock proteins inhibit in vitro A beta(1–42) amyloidogenesis. *FEBS Lett.* 416:117–121.
- Kumar-Singh, S., Dewachter, I., Moechars, D., Lubke, U., De Jonghe, C., Ceuterick, C., Checler, F., Naidu, A., Cordell, B., Cras, P., Van Broeckhoven, C., and Van Leuven, F. (2000). Behavioral disturbances without amyloid deposits in mice overexpressing human amyloid precursor protein with Flemish (A692G) or Dutch (E693Q) mutation. *Neurobiol. Dis.* 7:9–22.
- Lee, V.M., Goedert, M., and Trojanowski, J.Q. (2001). Neurodegenerative tauopathies. *Annu. Rev. Neurosci.* 24: 1121–1159.
- Leroy, E., Boyer, R., Auburger, G., Leube, B., Ulm, G., Mezey, E., Harta, G., Brownstein, M.J., Jonnalagada, S., Chernova, T., Dehejia, A., Lavedan, C., Gasser, T., Steinbach, P.J., Wilkinson, K.D., and Polymeropoulos, M.H. (1998). The ubiquitin pathway in Parkinson's disease. *Nature* 395:451–452.
- Levy-Lahad, E., Wijsman, E.M., Nemens, E., Anderson, L., Goddard, K.A., Weber, J.L., Bird, T.D., and Schellenberg, G.D. (1995). A familial Alzheimer's disease locus on chromosome 1. *Science* 269:970–973.
- Liang, J.J. (2000). Interaction between beta-amyloid and lens alphaB-crystallin. *FEBS Lett.* 484:98–101.
- Lindquist, S. (1986). The heat-shock response. *Annu. Rev. Biochem.* 55:1151–1191.
- Lindquist, S., and Kim, G. (1996). Heat-shock protein 104 expression is sufficient for thermotolerance in yeast. *Proc. Natl. Acad. Sci. USA* 93:5301–5306.

- Litt, M., Kramer, P., LaMorticella, D.M., Murphey, W., Lovrien, E.W., and Weleber, R.G. (1998). Autosomal dominant congenital cataract associated with a missense mutation in the human alpha crystallin gene CRYAA. *Hum. Mol. Genet.* 7:471–474.
- Lowenstein, D.H., Chan, P.H., and Miles, M.F. (1991). The stress protein response in cultured neurons: characterization and evidence for a protective role in excitotoxicity. *Neuron* 7:1053–1060.
- Luders, J., Demand, J., and Hohfeld, J. (2000). The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome. *J. Biol. Chem.* 275:4613–4617.
- Lue, L.F., Kuo, Y.M., Roher, A.E., Brachova, L., Shen, Y., Sue, L., Beach, T., Kurth, J.H., Rydel, R.E., and Rogers, J. (1999). Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. *Am. J. Pathol.* 155:853–862.
- Magrane, J., Smith, R.C., Walsh, K., and Querfurth, H.W. (2004). Heat shock protein 70 participates in the neuro-protective response to intracellularly expressed beta-amyloid in neurons. *J. Neurosci.* 24:1700–1706.
- Mangiarini, L., Sathasivam, K., Seller, M., Cozens, B., Harper, A., Hetherington, C., Lawton, M., Trotter, Y., Leach, H., Davies, S.W., and Bates, G.P. (1996). Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. *Cell* 87:493–506.
- Mangiarini, L., Sathasivam, K., Mahal, A., Mott, R., Seller, M., and Bates, G.P. (1997). Instability of highly expanded CAG repeats in mice transgenic for the Huntington's disease mutation. *Nat. Genet.* 15:197–200.
- Maraganore, D.M., Farrer, M.J., Hardy, J.A., Lincoln, S.J., McDonnell, S.K., and Rocca, W.A. (1999). Case-control study of the ubiquitin carboxy-terminal hydrolase L1 gene in Parkinson's disease. *Neurology* 53:1858–1860.
- Martindale, D., Hackam, A., Wiczorek, A., Ellerby, L., Wellington, C., McCutcheon, K., Singaraja, R., Kazemi-Esfarjani, P., Devon, R., Kim, S.U., Bredesen, D.E., Tufaro, F., and Hayden, M.R. (1998). Length of huntingtin and its polyglutamine tract influences localization and frequency of intracellular aggregates. *Nat. Genet.* 18: 150–154.
- McLean, C.A., Cherny, R.A., Fraser, F.W., Fuller, S.J., Smith, M.J., Beyreuther, K., Bush, A.I., and Masters, C.L. (1999). Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. *Ann. Neurol.* 46:860–866.
- McLean, P.J., Kawamata, H., and Hyman, B.T. (2001). Alpha-synuclein-enhanced green fluorescent protein fusion proteins form proteasome sensitive inclusions in primary neurons. *Neuroscience* 104:901–912.
- McLean, P.J., Kawamata, H., Shariff, S., Hewett, J., Sharma, N., Ueda, K., Breakefield, X.O. and Hyman, B.T. (2002). TorsinA and heat shock proteins act as molecular chaperones: suppression of alpha-synuclein aggregation. *J. Neurochem.* 83:846–854.
- Meriin, A.B., Zhang, X., He, X., Newnam, G.P., Chernoff, Y.O., and Sherman, M.Y. (2002). Huntington toxicity in yeast model depends on polyglutamine aggregation mediated by a prion-like protein Rnq1. *J. Cell Biol.* 157: 997–1004.
- Merry, D.E., Kobayashi, Y., Bailey, C.K., Taye, A.A., and Fischbeck, K.H. (1998). Cleavage, aggregation and toxicity of the expanded androgen receptor in spinal and bulbar muscular atrophy. *Hum. Mol. Genet.* 7:693–701.
- Minami, Y., Hohfeld, J., Ohtsuka, K., and Hartl, F.U. (1996). Regulation of the heat-shock protein 70 reaction cycle by the mammalian DnaJ homolog, Hsp40. *J. Biol. Chem.* 271:19617–19624.
- Mitraki, A., Betton, J.M., Desmadril, M., and Yon, J.M. (1987). Quasi-irreversibility in the unfolding-refolding transition of phosphoglycerate kinase induced by guanidine hydrochloride. *Eur. J. Biochem.* 163:29–34.
- Miyashita, T., Okamura-Oho, Y., Mito, Y., Nagafuchi, S., and Yamada, M. (1997). Dentatorubral pallidoluysian atrophy (DRPLA) protein is cleaved by caspase-3 during apoptosis. *J. Biol. Chem.* 272:29238–29242.
- Moechars, D., Dewachter, I., Lorent, K., Reverse, D., Baekelandt, V., Naidu, A., Tesseur, I., Spittaels, K., Haute, C.V., Checler, F., Godaux, E., Cordell, B., and Van Leuven, F. (1999). Early phenotypic changes in transgenic mice that overexpress different mutants of amyloid precursor protein in brain. *J. Biol. Chem.* 274:6483–6492.
- Morimoto, R.I., and Santoro, M.G. (1998). Stress-inducible responses and heat shock proteins: new pharmacologic targets for cytoprotection. *Nat. Biotechnol.* 16:833–838.
- Muchowski, P.J. (2002). Protein misfolding, amyloid formation, and neurodegeneration: a critical role for molecular chaperones? *Neuron* 35:9–12.
- Muchowski, P.J., Schaffar, G., Sittler, A., Wanker, E.E., Hayer-Hartl, M.K., and Hartl, F.U. (2000). Hsp70 and hsp40 chaperones can inhibit self-assembly of polyglutamine proteins into amyloid-like fibrils. *Proc. Natl. Acad. Sci. USA* 97:7841–7846.
- Muchowski, P.J., Ning, K., D'Souza-Schorey, C., and Fields, S. (2002). Requirement of an intact microtubule cytoskeleton for aggregation and inclusion body formation by a mutant huntingtin fragment. *Proc. Natl. Acad. Sci. USA* 99: 727–732.

- Murata, S., Chiba, T., and Tanaka, K. (2003). CHIP: a quality-control E3 ligase collaborating with molecular chaperones. *Int. J. Biochem. Cell Biol.* 35:572–578.
- Nollen, E.A., Garcia, S.M., van Haften, G., Kim, S., Chavez, A., Morimoto, R.I., and Plasterk, R.H. (2004). Genome-wide RNA interference screen identifies previously undescribed regulators of polyglutamine aggregation. *Proc. Natl. Acad. Sci. USA* 101:6403–6408.
- Paresce, D.M., Ghosh, R.N., and Maxfield, F.R. (1996). Microglial cells internalize aggregates of the Alzheimer's disease amyloid beta-protein via a scavenger receptor. *Neuron* 17:553–565.
- Petrucelli, L., Dickson, D., Kehoe, K., Taylor, J., Snyder, H., Grover, A., De Lucia, M., McGowan, E., Lewis, J., Prihar, G., Kim, J., Dillmann, W.H., Browne, S.E., Hall, A., Voellmy, R., Tsuboi, Y., Dawson, T.M., Wolozin, B., Hardy, J., and Hutton, M. (2004). CHIP and Hsp70 regulate tau ubiquitination, degradation and aggregation. *Hum. Mol. Genet.* 13:703–714.
- Poirier, M.A., Li, H., Macosko, J., Cai, S., Amzel, M., and Ross, C.A. (2002). Huntingtin spheroids and protofibrils as precursors in polyglutamine fibrilization. *J. Biol. Chem.* 277:41032–41037.
- Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E.S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W.G., Lazzarini, A.M., Duvoisin, R.C., Di Iorio, G., Golbe, L.I., and Nussbaum, R.L. (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 276:2045–2047.
- Prodromou, C., Roe, S.M., O'Brien, R., Ladbury, J.E., Piper, P.W., and Pearl, L.H. (1997). Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell* 90:65–75.
- Ratovitski, T., Corson, L.B., Strain, J., Wong, P., Cleveland, D.W., Culotta, V.C., and Borchelt, D.R. (1999). Variation in the biochemical/biophysical properties of mutant superoxide dismutase 1 enzymes and the rate of disease progression in familial amyotrophic lateral sclerosis kindreds. *Hum. Mol. Genet.* 8:1451–1460.
- Ravikumar, B., Duden, R., and Rubinstein, D.C. (2002). Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. *Hum. Mol. Genet.* 11:1107–1117.
- Roe, S.M., Prodromou, C., O'Brien, R., Ladbury, J.E., Piper, P.W., and Pearl, L.H. (1999). Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. *J. Med. Chem.* 42:260–266.
- Scherzinger, E., Lurz, R., Turmaine, M., Mangiarini, L., Hollenbach, B., Hasenbank, R., Bates, G.P., Davies, S.W., Lehrach, H., and Wanker, E.E. (1997). Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates in vitro and in vivo. *Cell* 90:549–558.
- Selkoe, D.J. (1999). Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature* 399:A23–A31.
- Selkoe, D.J. (2001). Alzheimer's disease: genes, proteins, and therapy. *Physiol. Rev.* 81:741–766.
- Sherman, M.Y., and Goldberg, A.L. (2001). Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. *Neuron* 29:15–32.
- Sherrington, R., Rogaev, E.I., Liang, Y., Rogaeva, E.A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., et al. (1995). Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* 375:754–760.
- Shimura, H., Miura-Shimura, Y., and Kosik, K.S. (2004a). Binding of tau to heat shock protein 27 leads to decreased concentration of hyperphosphorylated tau and enhanced cell survival. *J. Biol. Chem.* 279:17957–17962.
- Shimura, H., Schwartz, D., Gygi, S.P., and Kosik, K.S. (2004b). CHIP-Hsc70 complex ubiquitinates phosphorylated tau and enhances cell survival. *J. Biol. Chem.* 279:4869–4876.
- Shinder, G.A., Lacourse, M.C., Minotti, S., and Durham, H.D. (2001). Mutant Cu/Zn-superoxide dismutase proteins have altered solubility and interact with heat shock/stress proteins in models of amyotrophic lateral sclerosis. *J. Biol. Chem.* 276:12791–12796.
- Shorter, J., and Lindquist, S. (2004). Hsp104 catalyzes formation and elimination of self-replicating Sup35 prion conformers. *Science*.
- Singer, M.A., and Lindquist, S. (1998). Multiple effects of trehalose on protein folding in vitro and in vivo. *Mol. Cell.* 1:639–648.
- Singleton, A.B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., Hulihan, M., Peuralinna, T., Dutra, A., Nussbaum, R., Lincoln, S., Crawley, A., Hanson, M., Maraganore, D., Adler, C., Cookson, M.R., Muentert, M., Baptista, M., Miller, D., Blacato, J., Hardy, J., and Gwinn-Hardy, K. (2003). alpha-Synuclein locus triplication causes Parkinson's disease. *Science* 302:841.
- Sitler, A., Lurz, R., Lueder, G., Priller, J., Lehrach, H., Hayer-Hartl, M.K., Hartl, F.U., and Wanker, E.E. (2001). Geldanamycin activates a heat shock response and inhibits huntingtin aggregation in a cell culture model of Huntington's disease. *Hum. Mol. Genet.* 10:1307–1315.

- Smith, D.F., Whitesell, L., and Katsanis, E. (1998). Molecular chaperones: biology and prospects for pharmacological intervention. *Pharmacol. Rev.* 50:493–514.
- Soti, C., and Csermely, P. (2002). Chaperones and aging: role in neurodegeneration and in other civilizational diseases. *Neurochem. Int.* 41:383–389.
- Spillantini, M.G., Schmidt, M.L., Lee, V.M., Trojanowski, J.Q., Jakes, R., and Goedert, M. (1997). Alpha-synuclein in Lewy bodies. *Nature* 388:839–840.
- Stathopoulos, P.B., Rumfeldt, J.A., Scholz, G.A., Irani, R.A., Frey, H.E., Hallewell, R.A., Lepock, J.R., and Meiring, E.M. (2003). Cu/Zn superoxide dismutase mutants associated with amyotrophic lateral sclerosis show enhanced formation of aggregates in vitro. *Proc. Natl. Acad. Sci. USA* 100:7021–7026.
- Stege, G.J., Renkawek, K., Overkamp, P.S., Verschuure, P., van Rijk, A.F., Reijnen-Aalbers, A., Boelens, W.C., Bosman, G.J., and de Jong, W.W. (1999). The molecular chaperone alphaB-crystallin enhances amyloid beta neurotoxicity. *Biochem. Biophys. Res. Commun.* 262:152–156.
- Takata, K., Kitamura, Y., Tsuchiya, D., Kawasaki, T., Taniguchi, T., and Shimohama, S. (2003). Heat shock protein-90-induced microglial clearance of exogenous amyloid-beta1–42 in rat hippocampus in vivo. *Neurosci. Lett.* 344: 87–90.
- Takeuchi, H., Kobayashi, Y., Yoshihara, T., Niwa, J., Doyu, M., Ohtsuka, K., and Sobue, G. (2002). Hsp70 and Hsp40 improve neurite outgrowth and suppress intracytoplasmic aggregate formation in cultured neuronal cells expressing mutant SOD1. *Brain Res.* 949:11–22.
- Tanaka, M., Machida, Y., Niu, S., Ikeda, T., Jana, N.R., Doi, H., Kurosawa, M., Nekooki, M., and Nukina, N. (2004). Trehalose alleviates polyglutamine-mediated pathology in a mouse model of Huntington disease. *Nat. Med.* 10: 148–154.
- Tatzelt, J., Prusiner, S.B., and Welch, W.J. (1996). Chemical chaperones interfere with the formation of scrapie prion protein. *EMBO J.* 15:6363–6373.
- Tavaria, M., Gabriele, T., Kola, I., and Anderson, R.L. (1996). A hitchhiker's guide to the human Hsp70 family. *Cell Stress Chaperones* 1:23–28.
- Taylor, J.P., Tanaka, F., Robitschek, J., Sandoval, C.M., Taye, A., Markovic-Plese, S., and Fischbeck, K.H. (2003). Aggresomes protect cells by enhancing the degradation of toxic polyglutamine-containing protein. *Hum. Mol. Genet.* 12:749–757.
- Terry, R.D., Peck, A., DeTeresa, R., Schechter, R., and Horoupian, D.S. (1981). Some morphometric aspects of the brain in senile dementia of the Alzheimer type. *Ann. Neurol.* 10:184–192.
- The Huntington's Disease Collaborative Research Group. (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* 72:971–983.
- Tienari, P.J., Ida, N., Ikonen, E., Simons, M., Weidemann, A., Multhaup, G., Masters, C.L., Dotti, C.G., and Beyreuther, K. (1997). Intracellular and secreted Alzheimer beta-amyloid species are generated by distinct mechanisms in cultured hippocampal neurons. *Proc. Natl. Acad. Sci. USA* 94:4125–4130.
- Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A.R., Healy, D.G., Albanese, A., Nussbaum, R., Gonzalez-Maldonado, R., Deller, T., Salvi, S., Cortelli, P., Gilks, W.P., Latchman, D.S., Harvey, R.J., Dallapiccola, B., Auburger, G., and Wood, N.W. (2004). Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* 304:1158–1160.
- van Duijn, C.M., Dekker, M.C., Bonifati, V., Galjaard, R.J., Houwing-Duistermaat, J.J., Snijders, P.J., Testers, L., Breedveld, G.J., Horstink, M., Sandkuijl, L.A., van Swieten, J.C., Oostra, B.A., and Heutink, P. (2001). Park7, a novel locus for autosomal recessive early-onset parkinsonism, on chromosome 1p36. *Am. J. Hum. Genet.* 69: 629–634.
- Vicart, P., Caron, A., Guicheney, P., Li, Z., Prevost, M.C., Faure, A., Chateau, D., Chapon, F., Tome, F., Dupret, J.M., Paulin, D., and Fardeau, M. (1998). A missense mutation in the alphaB-crystallin chaperone gene causes a desmin-related myopathy. *Nat. Genet.* 20:92–95.
- Wacker, J.L., Zareie, M.H., Fong, H., Sarikaya, M., and Muchowski, P.J. (2004). Kinetic destabilization of pre-fibrillar polyglutamine intermediates by the molecular chaperones Hsp70/Hsp40. submitted.
- Waelter, S., Boeddrich, A., Lurz, R., Scherzinger, E., Lueder, G., Lehrach, H., and Wanker, E.E. (2001). Accumulation of mutant huntingtin fragments in aggresome-like inclusion bodies as a result of insufficient protein degradation. *Mol. Biol. Cell* 12:1393–1407.
- Wall, D., Zylicz, M., and Georgopoulos, C. (1994). The NH2-terminal 108 amino acids of the *Escherichia coli* DnaJ protein stimulate the ATPase activity of DnaK and are sufficient for lambda replication. *J. Biol. Chem.* 269: 5446–5451.
- Wall, D., Zylicz, M., and Georgopoulos, C. (1995). The conserved G/F motif of the DnaJ chaperone is necessary for the activation of the substrate binding properties of the DnaK chaperone. *J. Biol. Chem.* 270:2139–2144.

- Walsh, D.M., Klyubin, I., Fadeeva, J.V., Cullen, W.K., Anwyl, R., Wolfe, M.S., Rowan, M.J., and Selkoe, D.J. (2002). Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* 416:535–539.
- Wang, A., and Bolen, D.W. (1997). A naturally occurring protective system in urea-rich cells: mechanism of osmolyte protection of proteins against urea denaturation. *Biochemistry* 36:9101–9108.
- Wang, J., Dickson, D.W., Trojanowski, J.Q., and Lee, V.M. (1999). The levels of soluble versus insoluble brain Abeta distinguish Alzheimer's disease from normal and pathologic aging. *Exp. Neurol.* 158:328–337.
- Wanker, E.E. (2000). Protein aggregation and pathogenesis of Huntington's disease: mechanisms and correlations. *Biol. Chem.* 381:937–942.
- Warrick, J.M., Chan, H.Y., Gray-Board, G.L., Chai, Y., Paulson, H.L., and Bonini, N.M. (1999). Suppression of polyglutamine-mediated neurodegeneration in *Drosophila* by the molecular chaperone HSP70. *Nat. Genet.* 23: 425–428.
- Warrick, J.M., Paulson, H.L., Gray-Board, G.L., Bui, Q.T., Fischbeck, K.H., Pittman, R.N., and Bonini, N.M. (1998). Expanded polyglutamine protein forms nuclear inclusions and causes neural degeneration in *Drosophila*. *Cell* 93: 939–949.
- Watanabe, M., Dykes-Hoberg, M., Culotta, V.C., Price, D.L., Wong, P.C., and Rothstein, J.D. (2001). Histological evidence of protein aggregation in mutant SOD1 transgenic mice and in amyotrophic lateral sclerosis neural tissues. *Neurobiol. Dis.* 8:933–941.
- Webb, J.L., Ravikumar, B., Atkins, J., Skepper, J.N., and Rubinsztajn, D.C. (2003). Alpha-Synuclein is degraded by both autophagy and the proteasome. *J. Biol. Chem.* 278:25009–25013.
- Weissman, A.M. (2001). Themes and variations on ubiquitylation. *Nat. Rev. Mol. Cell. Biol.* 2:169–178.
- Wellington, C.L., Ellerby, L.M., Hackam, A.S., Margolis, R.L., Trifiro, M.A., Singaraja, R., McCutcheon, K., Salvesen, G.S., Propp, S.S., Bromm, M., Rowland, K.J., Zhang, T., Rasper, D., Roy, S., Thornberry, N., Pinsky, L., Kakizuka, A., Ross, C.A., Nicholson, D.W., Bredeisen, D.E., and Hayden, M.R. (1998). Caspase cleavage of gene products associated with triplet expansion disorders generates truncated fragments containing the polyglutamine tract. *J. Biol. Chem.* 273:9158–9167.
- Willingham, S., Outeiro, T.F., DeVit, M.J., Lindquist, S.L., and Muchowski, P.J. (2003). Yeast genes that enhance the toxicity of a mutant huntingtin fragment or alpha-synuclein. *Science* 302:1769–1772.
- Wirths, O., Multhaup, G., Czech, C., Blanchard, V., Moussaoui, S., Tremp, G., Pradier, L., Beyreuther, K., and Bayer, T.A. (2001). Intraneuronal Abeta accumulation precedes plaque formation in beta-amyloid precursor protein and presenilin-1 double-transgenic mice. *Neurosci. Lett.* 306:116–120.
- Wyttenbach, A., Sauvageot, O., Carmichael, J., Diaz-Latoud, C., Arrigo, A.P., and Rubinsztajn, D.C. (2002). Heat shock protein 27 prevents cellular polyglutamine toxicity and suppresses the increase of reactive oxygen species caused by huntingtin. *Hum. Mol. Genet.* 11:1137–1151.
- Yang, Y., Turner, R.S., and Gaut, J.R. (1998). The chaperone BiP/GRP78 binds to amyloid precursor protein and decreases Abeta40 and Abeta42 secretion. *J. Biol. Chem.* 273:25552–25555.
- Yoshida, H., Yoshizawa, T., Shibasaki, F., Shoji, S., and Kanazawa, I. (2002). Chemical chaperones reduce aggregate formation and cell death caused by the truncated Machado-Joseph disease gene product with an expanded polyglutamine stretch. *Neurobiol. Dis.* 10:88–99.
- Zhou, H., Li, S.H., and Li, X.J. (2001). Chaperone suppression of cellular toxicity of huntingtin is independent of polyglutamine aggregation. *J. Biol. Chem.* 276:48417–48424.
- Zoghbi, H.Y., and Orr, H.T. (2000). Glutamine repeats and neurodegeneration. *Annu. Rev. Neurosci.* 23:217–247.
- Zou, J., Guo, Y., Guettouche, T., Smith, D.F., and Voellmy, R. (1998). Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell* 94:471–480.

Mechanisms of Active Solubilization of Stable Protein Aggregates by Molecular Chaperones

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1. Abstract

Protein destabilization by mutations or external stresses may lead to misfolding and aggregation in the cell. Often, damage is not limited to a simple loss of function, but the hydrophobic exposure of aggregate surfaces may impair membrane functions and promote the aggregation of other proteins. Such a “proteinacious infectious” behavior is not limited to prion diseases. It is associated to most protein-misfolding neurodegenerative diseases and to aging in general. With the molecular chaperones and proteases, cells have evolved powerful tools that can specifically recognize and act upon misfolded and aggregated proteins. Whereas some chaperones passively prevent aggregate formation and propagation, others actively unfold and solubilize stable aggregates. In particular, ATPase chaperones and proteases serve as an intracellular defense network that can specifically identify and actively remove by refolding or degradation potentially infectious cytotoxic aggregates. Here we discuss two types of molecular mechanisms by which ATPase chaperones may actively solubilize stable aggregates: (1) unfolding by power strokes, using the Hsp100 ring chaperones, and (2) unfolding by random movements of individual Hsp70 molecules. In bacteria, fungi, and plants, the two mechanisms are key for reducing protein damages from abiotic stresses. In animals devoid of Hsp100, Hsp70 appears as the core element of the chaperone network, preventing the formation and actively removing disease-causing protein aggregates.

2. Choosing Between Native Folding and Misfolding

Anfinsen (1973) demonstrated that polypeptides artificially unfolded by urea or Guanidium-HCl, can spontaneously refold into native structures upon removal of the denaturing agent. This established that the primary sequence of a protein contains all the necessary information for its spontaneous acquisition of a native three-dimensional structure (Anfinsen, 1973). This general principle was confirmed with various model proteins under optimal *in vitro* conditions, such as low protein concentrations, low temperatures, and the presence of viscous compatible solutes (Viitanen *et al.*, 1990; Diamant *et al.*, 2001, 2003). However, under unfavorable conditions, such as elevated temperatures and elevated molecular crowding as in the cell (Martin and Hartl, 1997; van den Berg *et al.*, 1999), stable inactive structures generally referred to as aggregates, spontaneously form *in vitro* (Anfinsen, 1973) and in the cell (Parsell *et al.*, 1994; Kopito, 2000). In the case of most proteins,

thermodynamically, the native state is likely to be the most stable one. Yet, during and following stressful conditions, nearly as stable misfolded and misassembled (aggregated) states may also be spontaneously reached (Anfinsen, 1973).

Most *de novo* synthesized, or newly translocated proteins tend to reach a native three-dimensional structure readily during translation or translocation. *De novo* protein folding is, however, prone to errors and may require the presence of molecular chaperones such as *Escherichia coli* DnaK, GroEL, and Trigger factor at the ribosome exit, or as part of the import machinery into organelles (Deuerling *et al.*, 1999; Neupert and Brunner, 2002). In the case of protein import and refolding into the endoplasmic reticulum, mitochondria, and chloroplasts, Hsp70 (Neupert and Brunner, 2002), Bip (Corsi and Schekman, 1997), and possibly Hsp100 chaperones (Nielsen *et al.*, 1997) act as pulling and unfolding import motors, as well as posttranslocation refolding chaperones.

During their lifetime, natively folded proteins are subject to various abiotic stresses affecting their stability (Jaenicke, 1995). Under heat-shock, soluble native proteins that normally sequester hydrophobic residues away from water-exposed surfaces may locally melt and lose some of their secondary and tertiary structures (Ben-Zvi and Goloubinoff, 2002). To minimize hydrophobic interactions with water, unfolding intermediates readily seek alternative structures by forming intra- and intermolecular associations of exposed hydrophobic segments (Jaenicke, 1995). Although individual hydrophobic bonds are poorly specific and therefore may only feebly contribute to aggregate cohesiveness, a large number of such bonds may cooperate to form highly cohesive surfaces within, and among the misfolded polypeptides of an aggregate (Jaronec *et al.*, 2002; Perutz *et al.*, 2002). By virtue of the chemical equilibrium, which depends on the freedom of movement of each misfolded unit, the probability that individual misfolded monomers can spontaneously dissociate exponentially decreases as the number of cooperative hydrophobic bonds increases. We therefore suggest to use the term "stable" for protein aggregates whose intersubunit cohesiveness does not permit a significant dissociation of refoldable monomers, even when extremely diluted, within a biologically relevant time scale. Thus, very stable protein aggregates in the form of toxic inclusion bodies and amyloids may accumulate in the cell (Parsell *et al.*, 1994), unless specific cellular factors are present that can specifically identify, actively remove, and recycle them into nontoxic native or degraded forms (Yamamoto *et al.*, 2000).

Such factors are the cellular network of molecular chaperones and proteases that collaborate to protect native proteins from stress damages, prevent the formation of misfolded aggregates, scavenge, and solubilize already formed misfolded species, and finally, to refold or degrade aggregates into nontoxic protein species (Martin-Aparicio *et al.*, 2001; Sakahira *et al.*, 2002).

3. Many Molecular Chaperones Can Prevent Protein Aggregation

GroEL was initially shown *in vitro* to form a strong binary complex with urea-unfolded RubisCO, thus preventing RubisCO aggregation (Goloubinoff *et al.*, 1989a). Similarly, spontaneous GroEL-binding reduced the light-scattering signal of several model proteins undergoing aggregation (Buchner *et al.*, 1991; Holl *et al.*, 1991). Aggregation prevention by passive chaperone binding is now generally considered to be the common molecular mechanism shared by the different classes of molecular chaperones (Ellis, 2001; Hartl and Hayer-Hartl, 2002). Yet, although most chaperones can indeed passively prevent protein aggregation *in vitro* (Goloubinoff *et al.*, 1989a; Buchner *et al.*, 1991; Horwitz, 1992; Wiech *et al.*, 1992; Schröder *et al.*, 1993; Cyr, 1995), not all can or need to prevent aggregation to act as molecular chaperones. For example, Hsp100 and Trigger Factor (TF) do not significantly inhibit protein aggregation *in vitro* (Glover and Lindquist, 1998; Schaffitzel *et al.*, 2001).

However, both are important chaperones that lead to correct protein folding. Therefore, prevention of aggregation cannot serve as sole criterion to test for chaperone activity.

4. Some ATPase Chaperones Can Solubilize and Reactivate Stable Protein Aggregates

The *E. coli* chaperone DnaK was initially shown to assist the *in vitro* the reactivation of pre-formed heat-inactivated RNA polymerase in an ATP-dependent manner (Skowrya *et al.*, 1990). A decade later, using a combination of the yeast ATPase chaperones Hsp70 and Hsp104, Glover and Lindquist (1998) confirmed that heat- and Guanidium-HCl inactivated model proteins can be reactivated *in vitro*, albeit at very low efficiency (Glover and Lindquist, 1998). In contrast, the homologous chaperones from *E. coli*, ClpB, DnaK, DnaJ, and GrpE, displayed a very efficient *in vitro* disaggregation activity for variety of stable preformed protein aggregates, including aggregates from total cell extracts (Goloubinoff *et al.*, 1999; Mogk *et al.*, 1999). Efficient chaperone-mediated disaggregation of endogenous and recombinant protein aggregates was also confirmed *in vivo* (Goloubinoff *et al.*, 1989b; Mogk *et al.*, 1999; Tomoyasu *et al.*, 2001).

Whereas the *in vitro* disaggregation of large turbid aggregates required both ClpB and DnaK, disaggregation of small soluble aggregates and their subsequent reactivation required only the DnaK system, albeit in excess stoichiometric amounts compared to the substrate (Diamant *et al.*, 2000; Ben-Zvi and Goloubinoff, 2001). This suggested the Hsp70 is the core of the chaperone disaggregating machinery and explained how organisms lacking Hsp100, such as insects and mammals, can solubilize stable protein aggregates when expressing high concentrations of Hsp70 (Diamant *et al.*, 2000; Ben-Zvi and Goloubinoff, 2001).

5. Prevention of Aggregation Is Not Required for Chaperone-Dependent Protein Refolding

When substrates are bound to a molecular chaperone devoid of ATPase activity, such as the small HSPs, or the GroEL minichaperones (Zahn *et al.*, 1996a), they are not necessarily reactivated upon chaperone release (Ben-Zvi *et al.*, 1998; Veinger *et al.*, 1998). Rather, chaperone-bound denatured proteins tend to dissociate and aggregate, unless they are transferred to an ATP-hydrolysing chaperone, such as DnaK and GroEL (Veinger *et al.*, 1998; Ben-Zvi and Goloubinoff, 2001). Hence, although chaperone binding is important because it can reduce the extent of protein aggregation, it not able per se to restore stable misfolded structures into correct ones, within a biologically relevant time scale, without input of external energy.

6. ATPase Chaperones Can Unfold Misfolded Proteins

Chaperones such as DnaK, ClpB, or GroEL can “assist” the refolding of thousands of different proteins in the same cell, as well as foreign recombinant proteins (Goloubinoff *et al.*, 1989b; Mogk *et al.*, 1999; Ellis, 2000). It is therefore impossible that chaperones contribute specific structural information to each protein substrate, nor is this necessary: primary sequences contain all the necessary information for native folding (Anfinsen, 1973). However, under stress and crowded conditions, partially unfolded proteins may become alternatively stabilized by unspecific backbone interactions

(Fandrich and Dobson, 2002) leading to aggregates in which the primary sequence is prevented from dictating the tertiary structure. To assist so many different misfolded proteins using the same mechanism, molecular chaperones simply have to redirect them into the correct spontaneous refolding pathway, by converting stable misfolded conformers into less stable transient species that can spontaneously refold into the native structure. The *in vitro* experiments by Anfinsen suggests that such forms must be completely unfolded (Anfinsen, 1973), or at least locally unfolded polypeptides allowing the local formation of native secondary structures. Because in this process, a stable misfolded conformation has to be rapidly (within a biologically relevant time scale) converted into an unstable unfolded conformation, a significant energy must be introduced into the substrate by the chaperone. It is not surprising that only molecular chaperones capable of hydrolyzing ATP are implicated in the productive unfolding of stable misfolded proteins (Zhang *et al.*, 2002).

GroEL was the first chaperone to be suggested to act as an active unfoldase (Zahn *et al.*, 1996b). GroEL-dependent unfolding activity was demonstrated when ATP hydrolysis exposed otherwise protected hydrogen atoms in GroEL-bound misfolded RubisCO (Shtilerman *et al.*, 1999). The AAA+ proteins have also been implicated in protein unfolding. An ATP-dependent unfolding reaction of native-like SsrA-tagged substrate was demonstrated in several AAA+ family members, including ClpA, ClpX, the ATPase subunits of the proteasome, and of the proteasome homolog in *Archaea* (Weber-Ban *et al.*, 1999; Benaroudj and Goldberg, 2000; Hoskins *et al.*, 2000; Kim *et al.*, 2000; Singh *et al.*, 2000; Navon and Goldberg, 2001).

Hsp70 is also implicated in protein unfolding (Matouschek *et al.*, 1997; Frydman, 2001; Neupert and Brunner, 2002; Slepnev and Witt, 2002; Ben-Zvi *et al.*, 2004). The involvement of Hsp70, both in translocation and disaggregation, requires the active disruption of folded, or partially folded conformations of the protein precursors prior to translocation (Skowyra *et al.*, 1990; Matouschek *et al.*, 1997; Diamant *et al.*, 2000; Ben-Zvi and Goloubinoff, 2001). We therefore suggest to differentiate between molecular chaperones that can only bind to unfolded or misfolded polypeptides, thereby reducing the extent of misfolding and aggregation, from molecular chaperones that can actively unfold misfolded or alternatively folded proteins by consuming ATP. Both “prevention of aggregation” and active unfolding are complementary actions, because scavenging chaperones clearly benefit from less complex more readily accessible aggregates, which are stabilized on binding chaperones (Veinger *et al.*, 1998; Ben-Zvi and Goloubinoff, 2001).

7. Ring-Shaped Chaperone Oligomers Can Use Power Strokes to Actively Unfold Aggregates

Molecular motors, such as myosin, dynein, and kinesin have the ability to convert chemical energy stored in ATP into a conformational change, producing a mechanical working stroke (Schliwa and Woehlke, 2003). Ring oligomers, such as GroEL and hexameric AAA+ proteins like ClpB, present multiple protein-binding sites that can go apart from each other upon ATP-binding (Lee *et al.*, 2003). This can be translated into a power stroke pulling movement, capable of unfolding a bound misfolded protein. Dissociation of the chaperone may then allow the partially unfolded substrate to spontaneously acquire a more native conformation (see **Figure 8.2-1**; see color insert).

ATP binding by GroEL₁₄ rings was reported to induce a conformational rearrangement in the chaperonin oligomer, which increases the distance between the seven substrate-binding sites on the apical domains of the same ring (Roseman *et al.*, 1996; Xu *et al.*, 1997). At least three binding sites are required for efficient chaperonin-mediated reactivation (Farr *et al.*, 2000), suggesting that concerted movements within each GroEL₇ ring can provide a stretching stroke to a bound protein, induc-

ing local unfolding (Walter *et al.*, 1996; Zahn *et al.*, 1996b; Shtilerman *et al.*, 1999). Upon completion of the conformational change, the hydrophobic nature of the substrate binding sites is converted into hydrophilic (Xu *et al.*, 1997), leading to the dissociation of the substrate and allowing it to fold into its native conformation (Weissman *et al.*, 1995).

In the case of AAA+ rings, structural studies by cryo-electron microscopy of p97, a mammalian AAA+ protein (Rouiller *et al.*, 2002), and of bacterial ClpB (Lee *et al.*, 2003), showed a remarkable increase in the diameter of the N-terminal domain and in the protein-binding ring (see **Figure 8.2-1**; see color insert), between ADP and ATP bound structures. This strongly suggests that upon ATP-binding and hydrolysis, hexameric AAA+ proteins undergo a power-stroke movement. Similarly, the protease-associated ClpA chaperone and its protease-independent homologue, ClpB, may also use power-stroke movements to unfold protein aggregates. Indicative that ClpB can partially unfold preaggregated proteins, ClpB with ATP reduced the amount of β -sheet structures in aggregated proteins, as shown by Congo Red binding assays (Goloubinoff *et al.*, 1999). The ADP-binding form of ClpB has an apparent lower affinity for the aggregate than the ATP-binding form (Goloubinoff *et al.*, 1999), suggesting that following ATP hydrolysis, the partially unfolded substrate may be translocated across the ClpB ring (Weibezahn *et al.*, 2004) and/or dissociate from the chaperone. This would allow Hsp70-binding or rebinding, prevent aggregation, and actively unfold misfolded substrates into natively refoldable species (Ben-Zvi *et al.*, 2004; Mogk and Bukau, 2004).

8. Individual Chaperone Monomers Can Use Random Motions to Actively Unfold Aggregates

The molecular mechanism by which Hsp70 and its cochaperones can unfold and reactivate stable protein aggregates remains elusive (Horst *et al.*, 1997; Goloubinoff *et al.*, 1999; Diamant *et al.*, 2000; Neupert and Brunner, 2002). Unlike the ClpB and GroEL rings, DnaK has only one protein-binding site (Zhu *et al.*, 1996), and appears to act as an individual monomer (Schönfeld *et al.*, 1995; Watanabe and Yoshida, 2004) that cannot support coordinated power-stroke movements. The question remains by which energy-consuming mechanism can individually bound Hsp70 molecules achieve effective unfolding of stable misfolded proteins?

The spontaneous solubilization of aggregates can be favored under conditions that displace the chemical equilibrium towards dissociation. The local binding of an Hsp70 molecule to an exposed hydrophobic loop in an aggregate creates a unique situation in which the random motions of the bulky 70 kDa molecule, compared to limited dimensions of the loop, can transiently disrupt the hydrophobic bounds holding the flanking regions of the loop in a misfolded and collapsed state. In principle, it is thus possible for a single Hsp70 molecule to locally unfold small misfolded regions in aggregates, by recruiting the energy of ATP hydrolysis to carry transitions between the very high affinity “locked” state of the chaperone, during which unfolding by random motions may occur, with the low-affinity “unlocked” state, during which the newly unfolded segments can try to refold in a more native, less hydrophobically exposed conformation (Ben-Zvi *et al.*, 2004) (**Figure 8.2-2**; see color insert).

A similar Brownian ratchet mechanism has been suggested for Hsp70-mediated import of polypeptides into mitochondria (Horst *et al.*, 1997; Neupert and Brunner, 2002). As incoming polypeptides emerge from the import pore into the mitochondrial matrix, mitochondrial Hsp70 is thought to bind appearing hydrophobic segments. Brownian motions of the peptide-locked chaperone are then thought to drive the translocation of the polypeptide inward, allowing new hydrophobic segments to emerge and bind to new Hsp70s molecules. Hsp70 is thus proposed to act as a ratchet, capturing, actively unfolding, and driving the imported polypeptides into the organelle (Neupert and Brunner, 2002).

Because Hsp70 molecules have a very high degree of sequence conservation, it is unlikely that they use different mechanisms to mediate active protein translocation and solubilize stable protein aggregates. As in the case of mitochondrial import, it is likely that DnaK binds to exposed unfolded hydrophobic loops on the surface of aggregate-entangled polypeptides (Rudiger *et al.*, 1997) (**Figure 8.2-2a; see color insert**). Brownian motions of locked ADP-Hsp70s on the aggregated substrates or of incoming polypeptides from the import pores may induce local unfolding. When locked in the ADP-state to an emerging polypeptide, random chaperone agitations can be potentiated by a strong opposition (or reflection) of the much larger pore complex and the mitochondrial membrane. This can produce a strong unfolding/pulling force that can locally unwind the precursor and drive it inside the organelle (Neupert and Brunner, 2002). Similarly, the random movements of the 70kDa DnaK molecule against a much larger aggregate, can produce a strong local unfolding force that can be funnelled to the collapsed hydrophobic region of the flanking the Hsp70-binding sites in the aggregate (**Figure 8.2-2b; see color insert**). GrpE-mediated ADP release, inducing chaperone dissociation from the newly enlarged unfolded region (**Figure 8.2-2c; see color insert**) (Harrison *et al.*, 1997), can allow the spontaneous correct refolding of that region (**Figure 8.2-2d; see color insert**). Reiteration of such a local unfolding mechanism in other parts of the misfolded polypeptide could lead to the gradual refolding and disentanglement of the aggregate, resulting in the formation of a soluble native protein (Ben-Zvi *et al.*, 2004).

9. Conclusion: The Successive Lines of Defense Against Protein Aggregation and Diseases

Because protein aggregates are highly toxic (Zhu *et al.*, 2003), especially to cells carrying delicate membrane-dependant functions, like nervous influxes or photosynthesis, a set of defense mechanisms can intervene at different levels of damage and of stress severity. During a protein-denaturing stress, the first line of defence would be (I) protection by chemical chaperones that can accumulate, especially under osmotic stress, and by mechanisms depending on viscosity and water exclusion, stabilize native proteins in an active state, despite the ongoing stress (Diamant *et al.*, 2000, 2003). Similar to chemical chaperones, small HSPs display some ability to protect native proteins *in vivo* and *in vitro* (Forreiter *et al.*, 1997; Ben-Zvi and Goloubinoff, 2001). Remarkably, other types of molecular chaperones, including sophisticated ATP-hydrolyzing oligomeric machines, do not seem to protect native proteins against stress-induced inactivation *in vitro* and in the cell (Forreiter *et al.*, 1997).

Once labile proteins have escaped protection and are denatured, the second line of defense would be (II) prevention of aggregation by forming a stable complex with a binding chaperone, such as the small HSPs, Hsp60, Hsp40, Hsp70, or Hsp90. It is not known whether passive chaperone binding can also reduce the degree of intramolecular misfolding within individual polypeptides, but a prior reduction of protein aggregation clearly gives a thermodynamic advantage to subsequent unfolding/refolding actions by unfolding ATPase chaperones (Veinger *et al.*, 1998; Shitlerman *et al.*, 1999). Denatured proteins, which have been pre-bound *in vitro* to chaperones are more readily and efficiently refolded by the DnaK–DnaJ–GrpE system, compared to preaggregated proteins subsequently supplemented with the same chaperone system (Diamant and Goloubinoff, 1998; Diamant *et al.*, 2000).

Once denatured proteins have escaped protection by chemical chaperones, and have also escaped aggregation-prevention to form stable insoluble aggregates, the third line of defense would be (III) active unfolding and scavenging by ATP-chaperones such as Hsp100 and Hsp70 (Ben-Zvi *et al.*, 2004). Once unfolded and partially solubilized, the former aggregates may spontaneously refold into more native structures. However, various stresses, in particular oxygen radicals, can irreversibly

alter by oxidation or breakage of the polypeptide chain the folding information that is in the primary sequence. In such a case, nonrefoldable damaged proteins may recruit ATP-consuming unfolding chaperones in futile unfolding cycles. Consequently, when unfolding/refolding by ATPase chaperones has failed, the last line of defense is (IV) degradation. Hsp70 can direct E3 enzymes in the ubiquitination of damaged proteins for degradation by the proteasome. Similarly, ClpA or ClpX can direct damaged proteins for degradation by ClpP, leading to aggregate detoxification and residue recycling (Tomoyasu *et al.*, 2001; Jana and Nukina, 2003).

The overproduction or a better induction of disaggregating ATPase chaperones can halt the progression, and even cure, yeast prions and protein misfolding diseases in mammalian cells (Parsell *et al.*, 1994; Jana *et al.*, 2000; Sakahira *et al.*, 2002). This has far-reaching implications for the design of new strategies based on the expression of ATPase chaperones, to prevent the formation of cytotoxic aggregates and cure disease-causing amyloids and prions in mammals.

10. Abbreviation

HSP Heat-shock protein

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References

- Anfinsen, C.B. (1973). Principles that govern the folding of protein chains. *Science* 181:223–230.
- Benaroudj, N., and Goldberg, A.L. (2000). PAN, the proteasome-activating nucleotidase from archaeobacteria, is a protein-unfolding molecular chaperone. *Nat. Cell Biol.* 2:833–839.
- Ben-Zvi, A.P., and Goloubinoff, P. (2001). Review: mechanisms of disaggregation and refolding of stable protein aggregates by molecular chaperones. *J. Struct. Biol.* 135:84–93.
- Ben-Zvi, A.P., and Goloubinoff, P. (2002). Proteinaceous infectious behavior in non-pathogenic proteins is controlled by molecular chaperones. *J. Biol. Chem.* 277:49422–49427.
- Ben-Zvi, A.P., Chatellier, J., Fersht, A.R., and Goloubinoff, P. (1998). Minimal and optimal mechanisms for GroE-mediated protein folding. *Proc. Natl. Acad. Sci. USA* 95:15275–15280.
- Ben-Zvi A., De Los Rios, P., Dietler, G., and Goloubinoff, P. (2004). Active solubilization and refolding of stable protein aggregates by cooperative unfolding action of individual HSP70 chaperones. *J. Biol. Chem.* 279:37298–37303.
- Buchner, J., Schmidt, M., Fuchs, M., Jaenicke, R., Rudolph, R., Schmid, F.X., and Kiefhaber, T. (1991). GroE facilitates refolding of citrate synthase by suppressing aggregation. *Biochemistry* 30:1586–1591.
- Corsi, A.K., and Schekman, R. (1997). The luminal domain of Sec63p stimulates the ATPase activity of BiP and mediates BiP recruitment to the translocon in *Saccharomyces cerevisiae*. *J. Cell Biol.* 137:1483–1493.
- Cyr, M.D. (1995). Cooperation of the molecular chaperone Ydj1 with specific Hsp70 homologs to suppress protein aggregation. *FEBS Lett.* 359:129–132.
- Deuerling, E., Schulze-Specking, A., Tomoyasu, T., Mogk, A., and Bukau, B. (1999). Trigger factor and DnaK cooperate in folding of newly synthesized proteins. *Nature* 400:693–696.
- Diamant, S., and Goloubinoff, P. (1998). Temperature-controlled activity of DnaK-DnaJ-GrpE chaperones: protein-folding arrest and recovery during and after heat shock depends on the substrate protein and the GrpE concentration. *Biochemistry* 37:9688–9694.
- Diamant, S., Ben-Zvi, A.P., Bukau, B., and Goloubinoff, P. (2000). Size-dependent disaggregation of stable protein aggregates by the DnaK chaperone machinery. *J. Biol. Chem.* 275:21107–21113.

- Diamant, S., Eliahu, N., Rosenthal, D., and Goloubinoff, P. (2001). Chemical chaperones regulate molecular chaperones in vitro and in cells under combined salt and heat stresses. *J. Biol. Chem.* 276:39586–39591.
- Diamant, S., Rosenthal, D., Azem, A., Eliahu, N., Ben-Zvi, A.P., and Goloubinoff, P. (2003). Dicarboxylic amino acids and glycine-betaine regulate chaperone-mediated protein-disaggregation under stress. *Mol. Microbiol.* 49: 401–410.
- Ellis, R.J. (2000). Chaperone substrates inside the cell. *Trends Biochem. Sci.* 25:210–212.
- Ellis, R.J. (2001). Molecular chaperones: inside and outside the Anfinsen cage. *Curr. Biol.* 11:R1038–R1040.
- Fandrich, M., and Dobson, C.M. (2002). The behaviour of polyamino acids reveals an inverse side chain effect in amyloid structure formation. *EMBO J.* 21:5682–5690.
- Farr, G.W., Furtak, K., Rowland, M.B., Ranson, N.A., Saibil, H.R., Kirchhausen, T., and Horwich, A.L. (2000). Multivalent binding of nonnative substrate proteins by the chaperonin GroEL. *Cell* 100:561–573.
- Forreiter, C., Kirschner, M., and Nover, L. (1997). Stable transformation of an Arabidopsis cell suspension culture with firefly luciferase providing a cellular system for analysis of chaperone activity in vivo. *Plant Cell.* 9:2171–2181.
- Frydman, J. (2001). Folding of newly translated proteins in vivo: the role of molecular chaperones. *Annu. Rev. Biochem.* 70:603–647.
- Glover, J.R., and Lindquist, S. (1998). Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. *Cell* 94:73–82.
- Goloubinoff, P., Christeller, J.T., Gatenby, A.A., and Lorimer, G.H. (1989a). Reconstitution of active dimeric ribulose biphosphate carboxylase from an unfolded state depends on two chaperonin proteins and Mg-ATP. *Nature* 342: 884–889.
- Goloubinoff, P., Gatenby, A.A., and Lorimer, G.H. (1989b). GroE heat-shock proteins promote assembly of foreign prokaryotic ribulose biphosphate carboxylase oligomers in *Escherichia coli*. *Nature* 337:44–47.
- Goloubinoff, P., Mogk, A., Ben-Zvi, A.P., Tomoyasu, T., and Bukau, B. (1999). Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. *Proc. Natl. Acad. Sci. USA* 96:13732–13737.
- Harrison, C.J., Hayer-Hartl, M., Di Liberto, M., Hartl, F., and Kuriyan, J. (1997). Crystal structure of the nucleotide exchange factor GrpE bound to the ATPase domain of the molecular chaperone DnaK. *Science* 276:431–435.
- Hartl, F.U., and Hayer-Hartl, M. (2002). Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* 295:1852–1858.
- Holl, N.B., Rudolph, R., Schmidt, M., and Buchner, J. (1991). Reconstitution of a heat shock effect in vitro: influence of GroE on the thermal aggregation of alpha-glucosidase from yeast. *Biochemistry* 30:11609–11614.
- Horst, M., Azem, A., Schatz, G., and Glick, B.S. (1997). What is the driving force for protein import into mitochondria? *Biochim. Biophys. Acta* 1318:71–78.
- Horwitz, J. (1992). Alpha-crystallin can function as a molecular chaperone. *Proc. Natl. Acad. Sci. U A* 89: 10449–10453.
- Hoskins, J.R., Singh, S.K., Maurizi, M.R., and Wickner, S. (2000). Protein binding and unfolding by the chaperone ClpA and degradation by the protease ClpAP. *Proc. Natl. Acad. Sci. USA* 97:8892–8897.
- Jaenicke, R. (1995). Folding and association versus misfolding and aggregation of proteins. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 348:97–105.
- Jana, N.R., and Nukina, N. (2003). Recent advances in understanding the pathogenesis of polyglutamine diseases: involvement of molecular chaperones and ubiquitin-proteasome pathway. *J. Chem. Neuroanat.* 26:95–101.
- Jana, N.R., Tanaka, M., Wang, G., and Nukina, N. (2000). Polyglutamine length-dependent interaction of Hsp40 and Hsp70 family chaperones with truncated N-terminal huntingtin: their role in suppression of aggregation and cellular toxicity. *Hum. Mol. Genet.* 9:2009–2018.
- Jaroniec, C.P., MacPhee, C.E., Astrof, N.S., Dobson, C.M., and Griffin, R.G. (2002). Molecular conformation of a peptide fragment of transthyretin in an amyloid fibril. *Proc. Natl. Acad. Sci. USA* 99:16748–16753.
- Kim, Y.I., Burton, R.E., Burton, B.M., Sauer, R.T., and Baker, T.A. (2000). Dynamics of substrate denaturation and translocation by the ClpXP degradation machine. *Mol. Cell* 5:639–648.
- Kopito, R.R. (2000). Aggresomes, inclusion bodies and protein aggregation. *Trends Cell Biol.* 10:524–530.
- Lee, S., Sowa, M.E., Watanabe, Y.H., Sigler, P.B., Chiu, W., Yoshida, M., and Tsai, F.T. (2003). The structure of ClpB: a molecular chaperone that rescues proteins from an aggregated state. *Cell* 115:229–240.
- Martin, J., and Hartl, F.-U. (1997). The effect of macromolecular crowding on chaperonin-mediated protein folding. *Proc. Natl. Acad. Sci. USA* 94:1107–1112.
- Martin-Aparicio, E., Yamamoto, A., Hernandez, F., Hen, R., Avila, J., and Lucas, J.J. (2001). Proteasomal-dependent aggregate reversal and absence of cell death in a conditional mouse model of Huntington's disease. *J. Neurosci.* 21:8772–8781.

- Matouschek, A., Azem, A., Ratliff, K., Glick, B.S., Schmid, K., and Schatz, G. (1997). Active unfolding of precursor proteins during mitochondrial protein import. *EMBO J.* 16:6727–6736.
- Mogk, A., and Bukau, B. (2004). Molecular chaperones: structure of a protein disaggregase. *Curr. Biol.* 14:R78–R80.
- Mogk, A., Tomoyasu, T., Goloubinoff, P., Rudiger, S., Roder, D., Langen, H., and Bukau, B. (1999). Identification of thermolabile *Escherichia coli* proteins: prevention and reversion of aggregation by DnaK and ClpB. *EMBO J.* 18: 6934–6949.
- Navon, A., and Goldberg, A.L. (2001). Proteins are unfolded on the surface of the ATPase ring before transport into the proteasome. *Mol. Cell* 8:1339–1349.
- Neupert, W., and Brunner, M. (2002). The protein import motor of mitochondria. *Nat. Rev. Mol. Cell. Biol.* 3:555–565.
- Nielsen, E., Akita, M., Davila-Aponte, J., and Keegstra, K. (1997). Stable association of chloroplastic precursors with protein translocation complexes that contain proteins from both envelope membranes and a stromal Hsp100 molecular chaperone. *EMBO J.* 16:935–946.
- Parsell, D.A., Kowal, A.S., Singer, M.A., and Lindquist, S. (1994). Protein disaggregation mediated by heat-shock protein Hsp104. *Nature* 372:475–478.
- Perutz, M.F., Finch, J.T., Berriman, J., and Lesk, A. (2002). Amyloid fibers are water-filled nanotubes. *Proc. Natl. Acad. Sci. USA* 99:5591–5595.
- Roseman, A.M., Chen, S., White, H., Braig, K., and Saibil, H.R. (1996). The chaperonin ATPase cycle: mechanism of allosteric switching and movements of substrate-binding domains in GroEL. *Cell* 87:241–251.
- Rouiller, I., DeLaBarre, B., May, A.P., Weis, W.I., Brunger, A.T., Milligan, R.A., and Wilson-Kubalek, E.M. (2002). Conformational changes of the multifunction p97 AAA ATPase during its ATPase cycle. *Nat. Struct. Biol.* 9: 950–957.
- Rudiger, S., Buchberger, A., and Bukau, B. (1997). Interaction of Hsp70 chaperones with substrates. *Nat. Struct. Biol.* 4:342–349.
- Sakahira, H., Breuer, P., Hayer-Hartl, M.K., and Hartl, F.U. (2002). Molecular chaperones as modulators of polyglutamine protein aggregation and toxicity. *Proc. Natl. Acad. Sci. USA* 99:16412–16418.
- Schaffitzel, E., Rudiger, S., Bukau, B., and Deuerling, E. (2001). Functional dissection of trigger factor and DnaK: interactions with nascent polypeptides and thermally denatured proteins. *Biol. Chem.* 382:1235–1243.
- Schliwa, M., and Woehlke, G. (2003). Molecular motors. *Nature* 422:759–765.
- Schönfeld, H.-J., Schmidt, D., Schröder, H., and Bukau, B. (1995). The DnaK chaperone system of *Escherichia coli*: quaternary structures and interactions of the DnaK and GrpE components. *J. Biol. Chem.* 270:2183–2189.
- Schröder, H., Langer, T., Hartl, F.-U., and Bukau, B. (1993). DnaK, DnaJ, GrpE form a cellular chaperone machinery capable of repairing heat-induced protein damage. *EMBO J.* 12:4137–4144.
- Shtilerman, M., Lorimer, G.H., and Englander, S.W. (1999). Chaperonin function: folding by forced unfolding. *Science* 284:822–825.
- Singh, S.K., Grimaud, R., Hoskins, J.R., Wickner, S., and Maurizi, M.R. (2000). Unfolding and internalization of proteins by the ATP-dependent proteases ClpXP and ClpAP. *Proc. Natl. Acad. Sci. USA* 97:8898–8903.
- Skowrya, D., Georgopoulos, C., and Zylicz, M. (1990). The *E. coli* dnaK gene product, the hsp70 homolog, can reactivate heat-inactivated RNA polymerase in an ATP hydrolysis-dependent manner. *Cell* 62:939–944.
- Slepenkov, S.V., and Witt, S.N. (2002). The unfolding story of the *Escherichia coli* Hsp70 DnaK: is DnaK a holdase or an unfoldase? *Mol. Microbiol.* 45:1197–1206.
- Tomoyasu, T., Mogk, A., Langen, H., Goloubinoff, P., and Bukau, B. (2001). Genetic dissection of the roles of chaperones and proteases in protein folding and degradation in the *Escherichia coli* cytosol. *Mol. Microbiol.* 40:397–413.
- van den Berg, B., Ellis, R.J., and Dobson, C.M. (1999). Effects of macromolecular crowding on protein folding and aggregation. *EMBO J.* 18:6927–6933.
- Veinger, L., Diamant, S., Buchner, J., and Goloubinoff, P. (1998). The small heat-shock protein IbpB from *Escherichia coli* stabilizes stress-denatured proteins for subsequent refolding by a multichaperone network. *J. Biol. Chem.* 273:11032–11037.
- Viitanen, P.V., Lubben, T.H., Reed, J., Goloubinoff, P., O’Keefe, D.P., and Lorimer, G.H. (1990). Chaperonin-facilitated refolding of ribulosebiphosphate carboxylase and ATP hydrolysis by chaperonin 60 (groEL) are K⁺ dependent. *Biochemistry* 29:5665–5671.
- Walter, S., Lorimer, G.H., and Schmid, F.X. (1996). A thermodynamic coupling mechanism for GroEL-mediated unfolding. *Proc. Natl. Acad. Sci. USA* 93:9425–9430.
- Watanabe, Y.H., and Yoshida, M. (2004). Trigonal DnaK-DnaJ complex vs free DnaK and DnaJ; heat stress converts the former to the latter and only the latter can do disaggregation in cooperation with ClpB. *J. Biol. Chem.* 279: 15723–15727.

- Weber-Ban, E.U., Reid, B.G., Miranker, A.D., and Horwich, A.L. (1999). Global unfolding of a substrate protein by the Hsp100 chaperone ClpA. *Nature* 401:90–93.
- Weibezahn, J., Tessarz, P., Schlieker, C., Zahn, R., Maglica, Z., Lee, S., Zentgraf, H., Weber-Ban, E.U., Dougan, D.A., Tsai, F.T., Mogk, A., and Bukau, B. (2004) Thermotolerance requires refolding of aggregated proteins by substrate translocation through the central pore of ClpB. *Cell* 119:653–665.
- Weissman, J.S., Hohl, C.M., Kovalenko, O., Kashi, Y., Chen, S., Braig, K., Saibil, H.R., Fenton, W.A., and Horwich, A.L. (1995). Mechanism of GroEL action: productive release of polypeptide from a sequestered position under GroES. *Cell* 83:577–587.
- Wiech, H., Buchner, J., Zimmermann, R., and Jakob, U. (1992). Hsp90 chaperones protein folding in vitro. *Nature* 358: 169–170.
- Xu, Z., Horwich, A.L., and Sigler, P.B. (1997). The crystal structure of the asymmetric GroEL-GroES-(ADP)7 chaperonin complex. *Nature* 388:741–750.
- Yamamoto, A., Lucas, J.J., and Hen, R. (2000). Reversal of neuropathology and motor dysfunction in a conditional model of Huntington's disease. *Cell* 101:57–66.
- Zahn, R., Buckle, A.M., Perrett, S., Johnson, C.M., Corrales, F.J., Golbik, R., and Fersht, A.R. (1996a). Chaperone activity and structure of monomeric polypeptide binding domains of GroEL. *Proc. Natl. Acad. Sci. USA* 93:15024–15029.
- Zahn, R., Perrett, S., and Fersht, A.R. (1996b). Conformational states bound by the molecular chaperones GroEL and secB: a hidden unfolding (annealing) activity. *J. Mol. Biol.* 261:43–61.
- Zhang, X., Beuron, F., and Freemont, P.S. (2002). Machinery of protein folding and unfolding. *Curr. Opin. Struct. Biol.* 12:231–238.
- Zhu, M., Li, J., and Fink, A.L. (2003). The association of alpha-synuclein with membranes affects bilayer structure, stability, and fibril formation. *J. Biol. Chem.* 278:40186–40197.
- Zhu, X., Zhao, X., Burkholder, W.F., Gragerov, A., Ogata, C.M., Gottesman, M.E., and Hendrickson, W.A. (1996). Structural analysis of substrate binding by the molecular chaperone DnaK. *Science* 272:1606–1614.

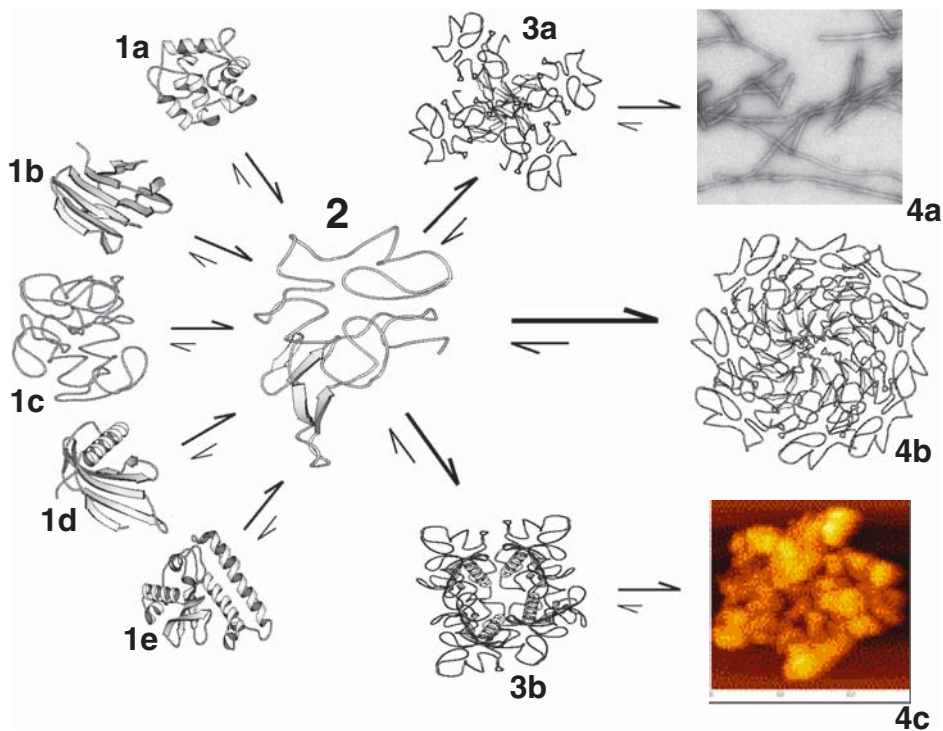


Figure 1-1. General model of protein aggregation. Aggregation is initiated by a structural transformation of proteins with different types of structure (α -helical, β -structural, natively unfolded, α + β or α / β ; single-domain or multidomain; etc., marked as 1a–1e) into the partially folded conformation (2). Partially folded molecules assemble into the specific oligomers (nucleus, or protofibrils, 3a and 3b). Depending on the peculiarities of amino acid sequence and environmental conditions protein may end in amyloid fibril, 4a, soluble oligomer, 4b, or amorphous aggregate, 4c. Figure adopted with the permission from (Uversky and Fink, 2004)

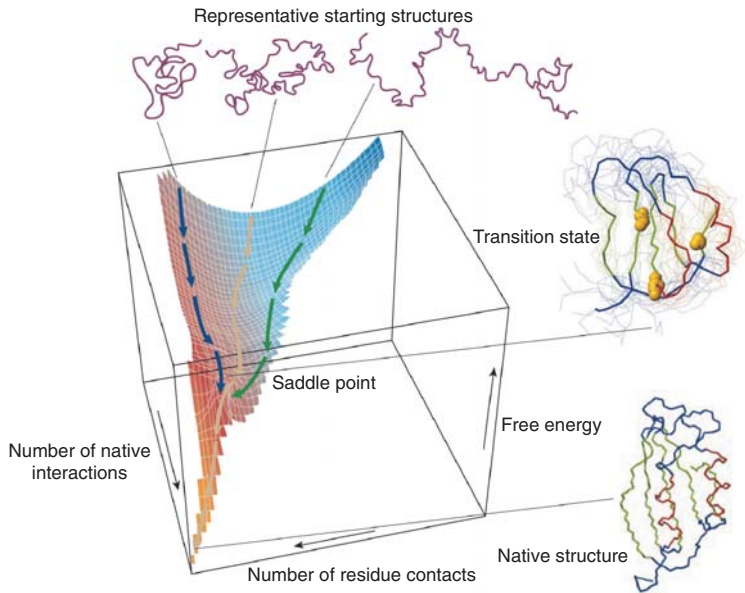


Figure 2-1. A schematic energy landscape for protein folding. This surface is derived from a computer simulation of the folding of a highly simplified model of a small protein. The surface “funnels” the multitude of denatured conformations to the unique native structure. The critical region in a simple surface such as this one is the saddle point corresponding to the transition state, the barrier that all molecules must cross if they are to fold to the native state. Superimposed on this schematic surface are ensembles of structures corresponding to different stages of the folding process. The transition state ensemble was calculated by using computer simulations constrained by experimental data from mutational studies of acylphosphatase. The yellow spheres in this ensemble represent the three “key residues” in the structure; when these residues have formed their native-like contacts the overall topology of the native fold is established. The structure of the native state is shown at the bottom of the surface; at the top are indicated schematically some contributors to the distribution of unfolded species that represent the starting point for folding. Also indicated on the surface are highly simplified trajectories for the folding of individual molecules (Dobson, 2003)

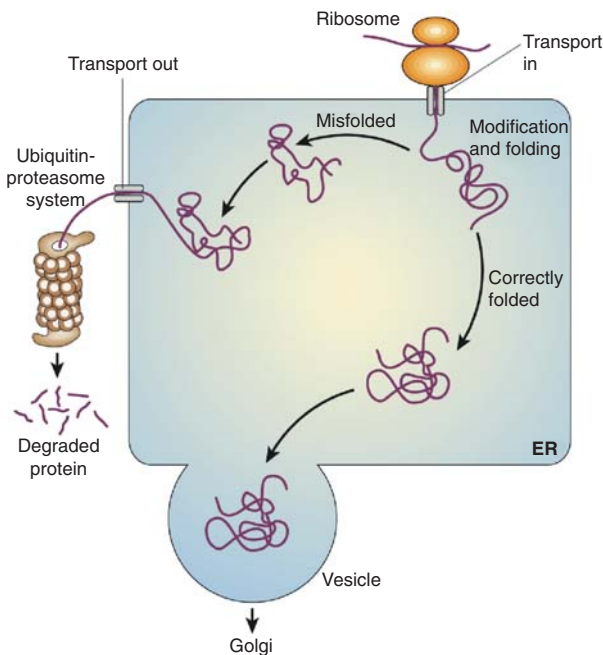


Figure 2-2. Folding in the ER. Newly synthesized proteins designed for export are transported into the ER where they undergo a series of modifications, such as glycosylation, and are helped to fold into their correct three-dimensional structures by molecular chaperones (not shown). Correctly folded proteins are then transported to the Golgi complex, encapsulated in membranes (called vesicles), and delivered to their final destination. Incorrectly folded proteins, however, are detected by a quality-control mechanism, retained in the ER, and sent along another pathway (the unfolded protein response) to be degraded by the ubiquitin–proteasome system (From Dobson, 2003, adapted from Kaufman, 2002.)

Figure 2-3. A molecular model of an amyloid fibril. This model is derived from cryo-EM analysis of fibrils grown from bovine insulin whose native topology is indicated in (a). The two chains of the insulin molecule (A, green and B, blue) are connected by three disulphide bridges (b). A possible topology for insulin in the fibrils is illustrated in (c), and (d) indicates how the β -strands could be assembled in a complete fibril, a model for which is shown in (e). This particular type of fibril consists of four “proto-filaments” that twist around one another to form a regular pattern with a diameter of approximately 10 nm (Jiménez *et al.*, 2002)

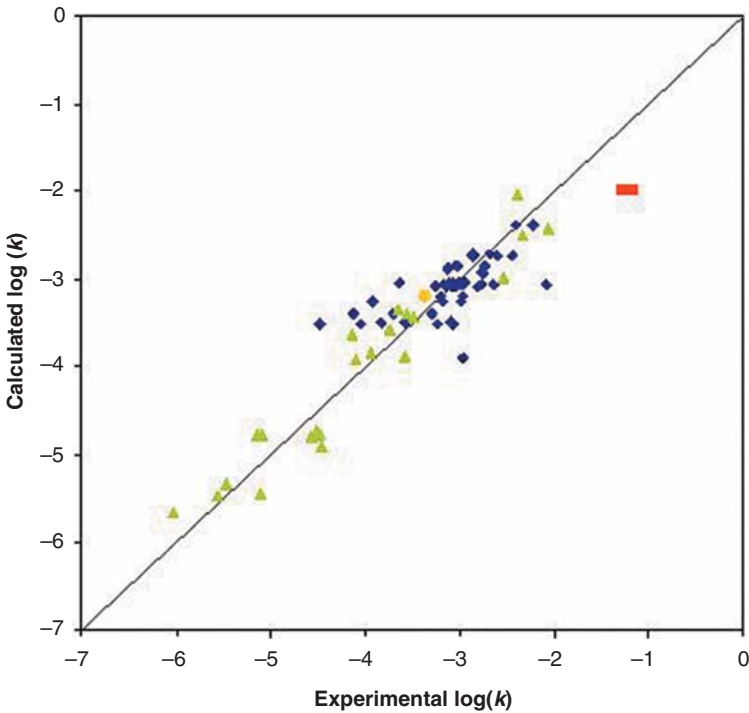
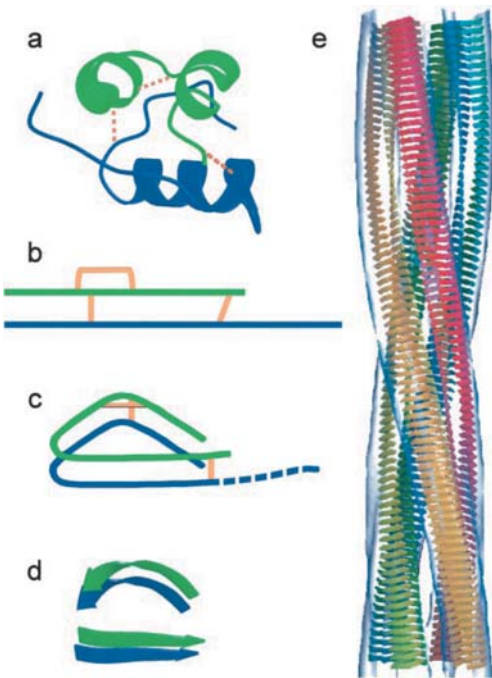


Figure 2-5. Plot of experimental aggregation rates for a series of peptides and proteins in their unfolded states against rates derived from an algorithm that incorporates intrinsic factors such as charge and hydrophobicity, and extrinsic factors such as pH and ionic strength. The points shown as blue diamonds are all for mutational variants of acylphosphatase (ACP), while those shown as green triangles are for other polypeptides. The red bar and yellow square are data for two systems that were not included in the data set used to generate the algorithm, and hence show its predictive value. (DuBay *et al.*, 2004)

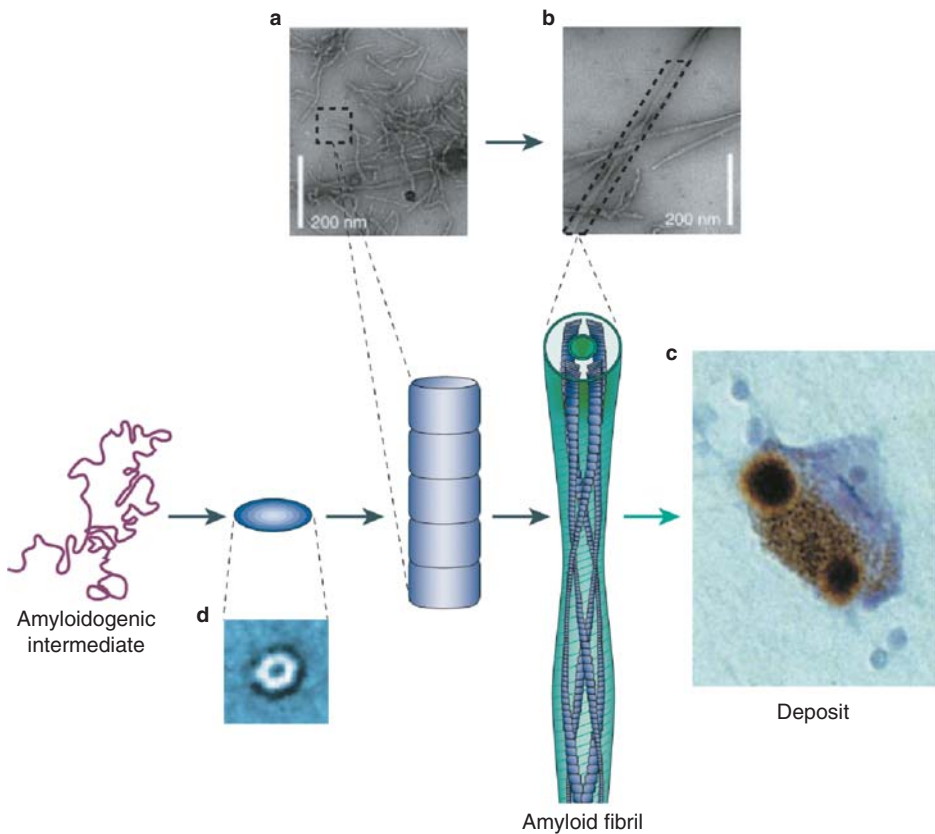


Figure 2-6. Schematic representation of the formation of amyloid fibrils and pathological deposits. An unfolded or partially unfolded peptide or protein initially forms small soluble aggregates that then assemble to form a variety of “prefibrillar” species, some or all of which can be toxic to living cells. These species undergo a series of additional assembly and reorganisational steps to give ordered amyloid fibrils of the type illustrated in Figure 12-3. The insets indicate images of the various species from electron microscopy (a) and (b) (Fändrich and Dobson, 2002), (c) optical microscopy (?), and (d) atomic force microscopy (Lashuel *et al.*, 2002). Pathological deposits such as the Lewy bodies associated with Parkinson’s disease, illustrated in (c), frequently contain large assemblies of fibrillar aggregates, along with other components including molecular chaperones (Dobson, 2003)

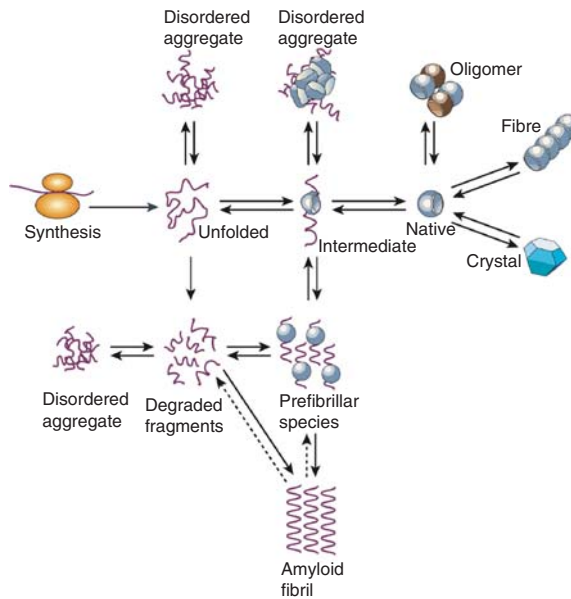


Figure 2-7. A unified view of some of the types of structure that can be formed by polypeptide chains. An unstructured chain, for example newly synthesized on a ribosome, can fold to a monomeric native structure, often through one or more partly folded intermediates. It can, however, experience other fates such as degradation or aggregation. An amyloid fibril is just one form of aggregate, but it is unique in having a highly organized “misfolded” structure. Other assemblies, including functional oligomers, macromolecular complexes, and natural protein fibres, contain natively folded molecules, as do the protein crystals produced *in vitro* for X-ray diffraction studies of their structures. The populations and inter-conversions of the various states are determined by their relative thermodynamic and kinetic stabilities under any given conditions. In living systems, however, transitions between the different states are highly regulated by the environment and by the presence of molecular chaperones, proteolytic enzymes, and other factors. Failure of such regulatory mechanisms is likely to be a major factor in the onset and development of misfolding diseases (Dobson, 2003)

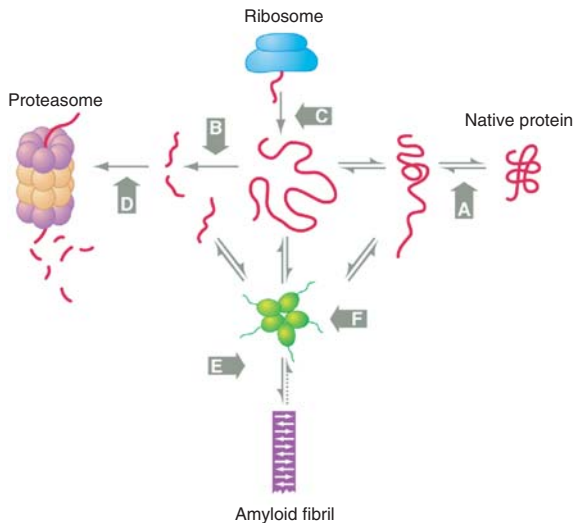


Figure 2-8. Therapeutic intervention in amyloid diseases. The conversion of normal soluble peptides and proteins into insoluble aggregates that are deposited in a variety of tissues is shown (Dobson, 2003). Highlighted are stages in the aggregation process where therapeutic intervention may be able to prevent or reverse aggregation. Therapeutic strategies include (A) stabilizing the native state; (B) inhibiting enzymes that process proteins into peptides with a propensity to aggregate; (C) altering protein synthesis; (D) stimulating clearance of misfolded proteins, for example, by boosting their proteasomal degradation; (E) inhibiting fibril assembly; (F) preventing accumulation of fibril precursors (Dobson, 2004)

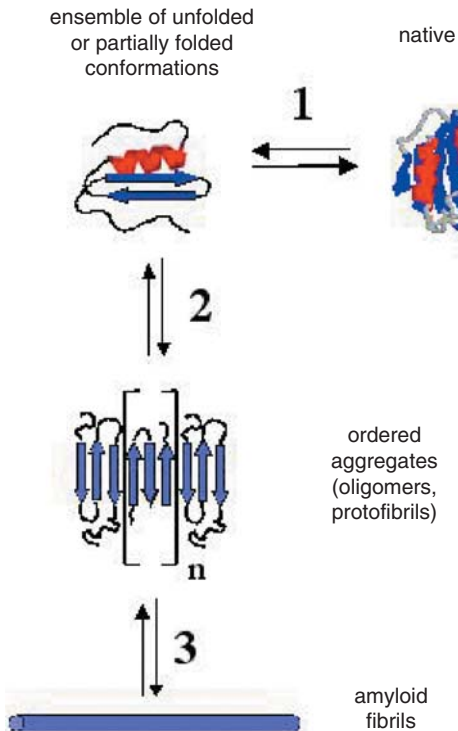


Figure 3-1. Schematic representation of amyloid fibril formation for a globular protein. The protein first unfolds at least partially and produce an ensemble of unfolded or partially folded conformations (step 1). Protein molecules in such a conformational state aggregate to produce nonfibrillar assemblies, generally referred to as oligomers or protofibrils (step 2), which subsequently convert into fibrils (step 3). The fibril formation process for relatively unstructured peptides or natively unfolded proteins such as A β , α -syn, or τ occurs directly via steps 2 and 3

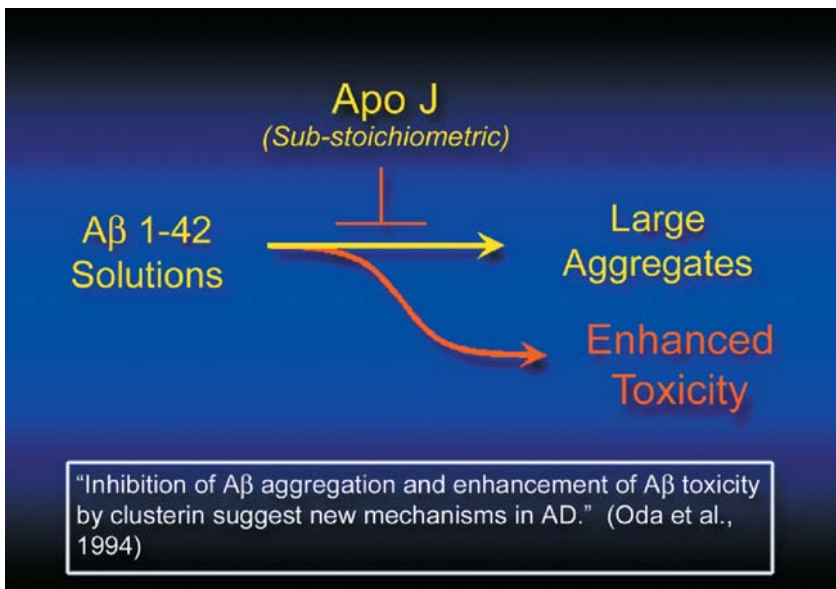


Figure 4-1. Early clue that subfibrillar species of amyloidogenic proteins could be toxic. The amyloidogenic peptide A β at first was thought to be neurotoxic only after formation of large fibrils. However, the chaperone-like action of apoJ (also known as clusterin), while drastically lowering the accumulation of large A β fibrils, actually increased neurotoxicity

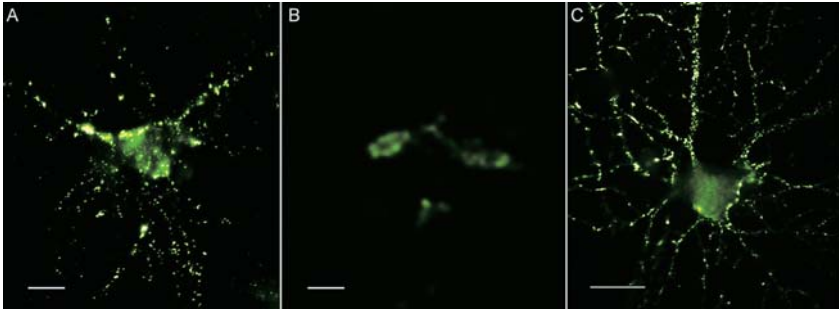


Figure 4-2. Small soluble A β oligomers act as synaptic ligands, a possible mechanism for the specific loss of memory in early AD. Soluble A β oligomers, whether obtained from AD brain tissue or prepared *in vitro*, bind at specific “hot spots” on differentiated hippocampal neurons (in culture), which have been identified as synaptic terminals. (A) Soluble extract from AD brain. (B) Soluble extract from control brain. (C) Oligomers prepared *in vitro*. Distribution shown by immunofluorescence microscopy using oligomer-selective antibodies. (From Gong *et al.*, 2003.)

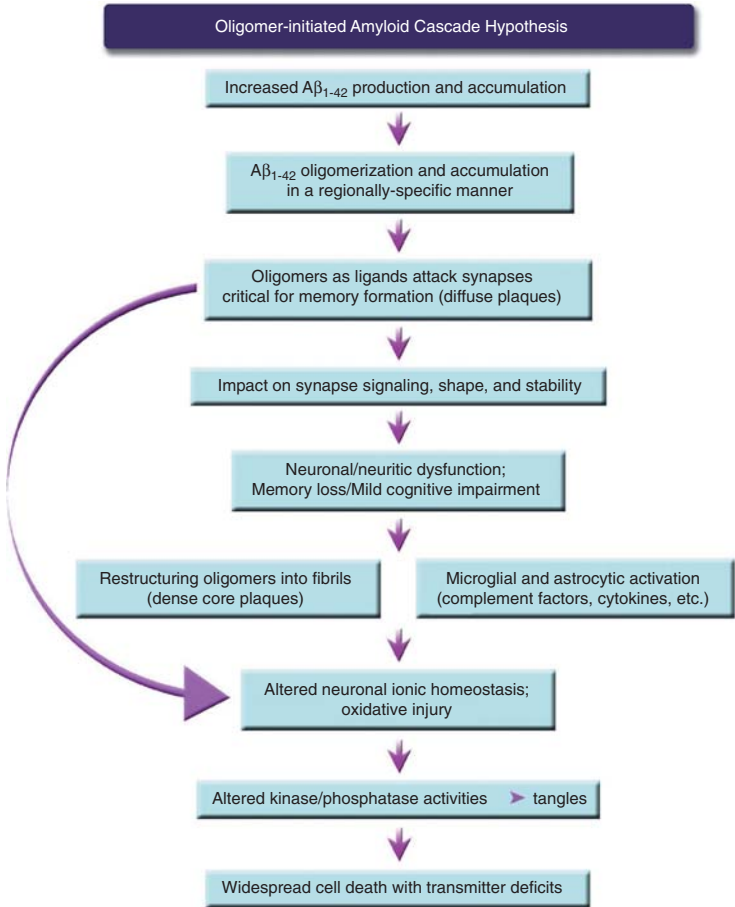


Figure 4-3. Updated version of the classic amyloid cascade hypothesis for Alzheimer’s disease. (Adapted from Hardy and Selkoe, 2002.)

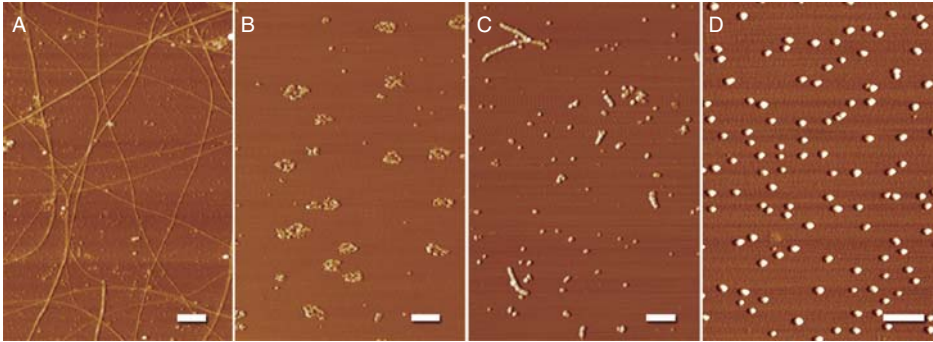


Figure 4-4. Atomic force microscopy shows that solutions of A β 42 peptide can yield assemblies of widely varying structure. Depending on solution conditions, A β 42 can yield (A) fibrils, (B) pore-like structures, (C) mixtures of small oligomers and protofibrils, or (D) pure globular oligomers. (Adapted from Chromy *et al.*, 2003.)

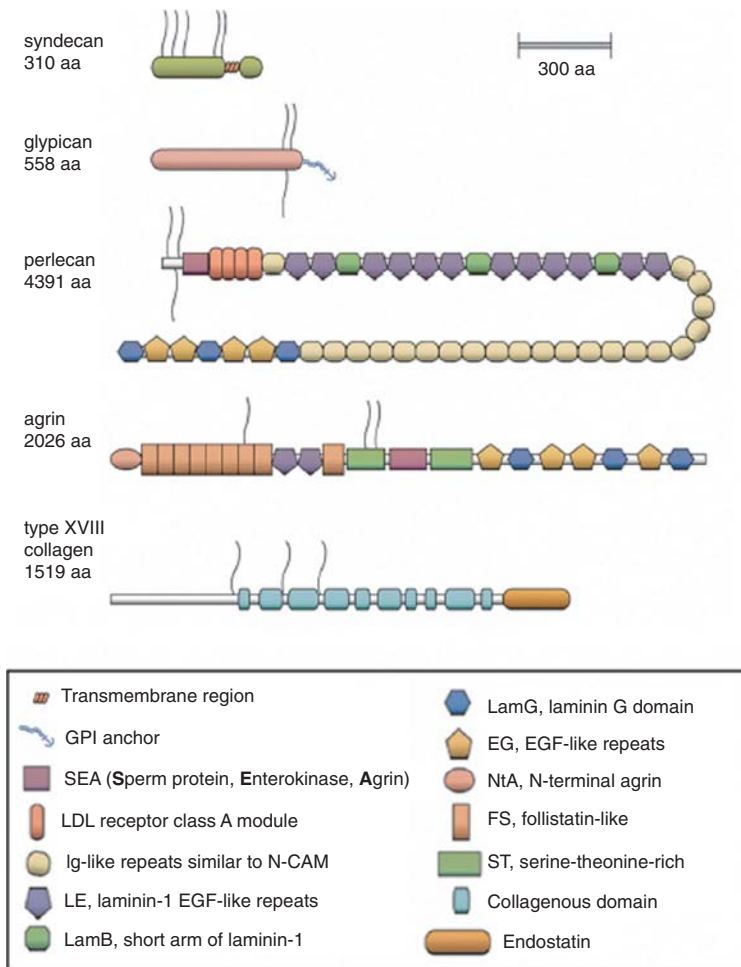


Figure 5-2. Structures of the five classes of HSPG. Syndecans are transmembrane HSPGs, while glypicans are membrane-associated as a result of a GPI anchor on the protein core. Perlecan and collagen XVIII are secreted HSPGs and components of the extracellular matrix. Agrin exists both as a transmembrane and secreted extracellular matrix HSPG. It can be seen that these HSPGs share some protein motifs that contribute to their function. The putative attachment sites for HS-GAG chains are also indicated. (Republished with permission of the American Society for Clinical Investigation, from Iozzo, 2001.)

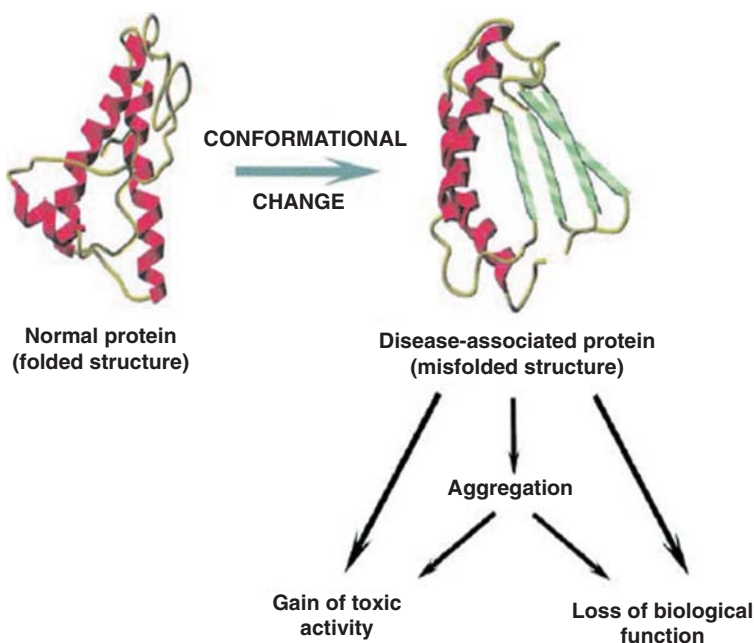


Figure 5-3. Schematic model illustrating how conformational changes in proteins can lead to misfolding and the introduction of significant β -sheet structure in proteins associated with amyloid and protein conformation disorder diseases. The misfolded protein then undergoes aggregation, with consequent loss of normal biological function or gain of toxic activity. (Reprinted by permission of Federation of the European Biochemical Societies from Soto, 2001.)

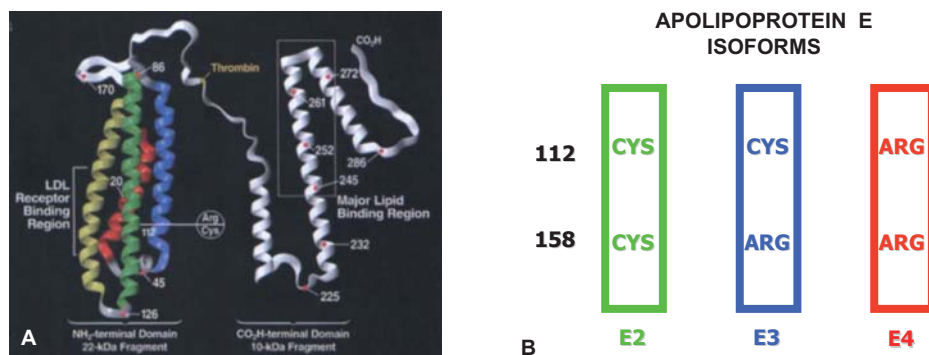


Figure 6-1. (A) Three-dimensional structure of apolipoprotein E as an example of protein class. Thrombin cleavage separates two domains: NH₂-terminal domain containing four class G amphipathic helices, and CO₂-terminal domain with class A amphipathic helices. The NH₂-terminal domain contains regions for the LDL receptor binding and possibly for interacting with A β . The CO₂-terminal domain is a principal lipid binding domain of apo E. (From Huang *et al.* 2001, with permission.) (B) Apo E isoforms types. Arginines in positions 112 and 158 of apo E4 allow for NH₂- and CO₂-terminal domain interaction, which is unique for this isoform

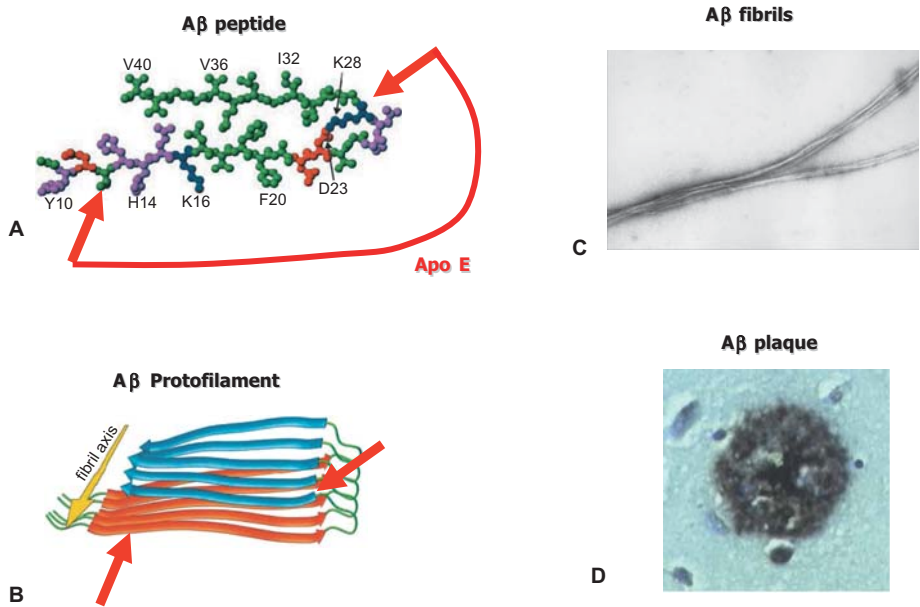


Figure 6-2. Putative mechanism of apo E involvement in Aβ fibril formation. (A,B) Model of Aβ fibrils formed by synthetic Aβ peptides as demonstrated by the solid-phase NMR technique. Apo E binds to 12–28 residues of Aβ stabilizing the β-turn, critical for bringing two hydrophobic domains (indicated by green color) together, which, in consequence, allows for assembly of single peptides in a protofilament. (Adapted from Petkova *et al.*, 2002, with permission.) (C) Electron micrograph of Aβ fibrils, and (D) a classical Aβ plaque from a brain of AD patient stained with 4G8 monoclonal antibody

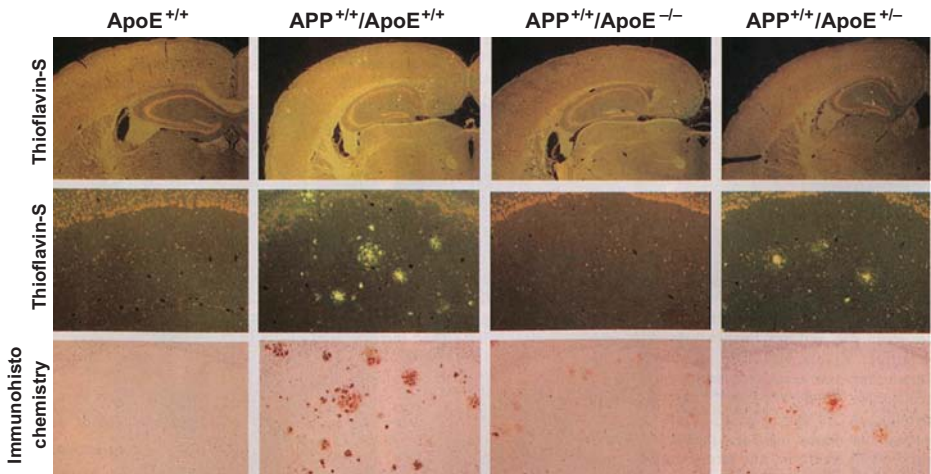


Figure 6-4. Apo E KO mice were used to demonstrate a critical role of apo E in deposition of Aβ in AD. The tg mice overexpressing the V717F APP mutant accumulate fibrillar Aβ deposits detected by both Thioflavin-S and immunohistochemistry. When these mice were crossbred with apo E KO mice, the V717F APP^{+/+}/ApoE^{-/-} strain had very limited Aβ deposition and virtually no fibrillar Aβ as demonstrated by Thioflavin-S staining. Mice with one copy of apoE (V717F APP^{+/+}/ApoE^{+/-}) showed an intermediate level of pathology. No amyloid deposits were found in wt mice (apoE^{+/+}). (From Bales *et al.*, 1997, with permission.)

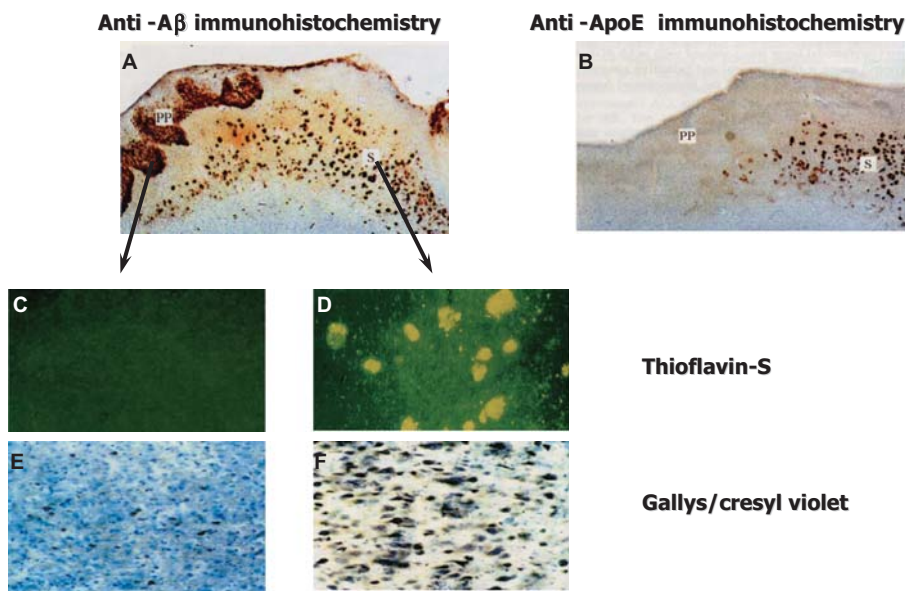


Figure 6-5. Apo E is essential for Aβ toxicity. (A) Numerous Aβ plaques in the subiculum (S) and confluent Aβ deposits covering over 90% of the presubiculum parvocellulare in the end-stage AD patient (6E10 monoclonal antibody reacting with 1–16 residues of Aβ). (B) Anti-apo E immunohistochemistry demonstrates the presence of apo E in subicular plaques but not in confluent presubicular deposits. Plaques in the subiculum contain fibrillar Aβ (D) as demonstrated by Thioflavin-S staining, whereas the presubiculum Aβ does not fibrillize even in the end stage of AD (C). It is the subiculum that suffers from neuronal loss and advance neurofibrillira pathology (F), whereas neuronal damage and number of neurofibrillary tangles in the presubiculum is mninimal. (From Wisniewski *et al.*, 1998, with permission.)

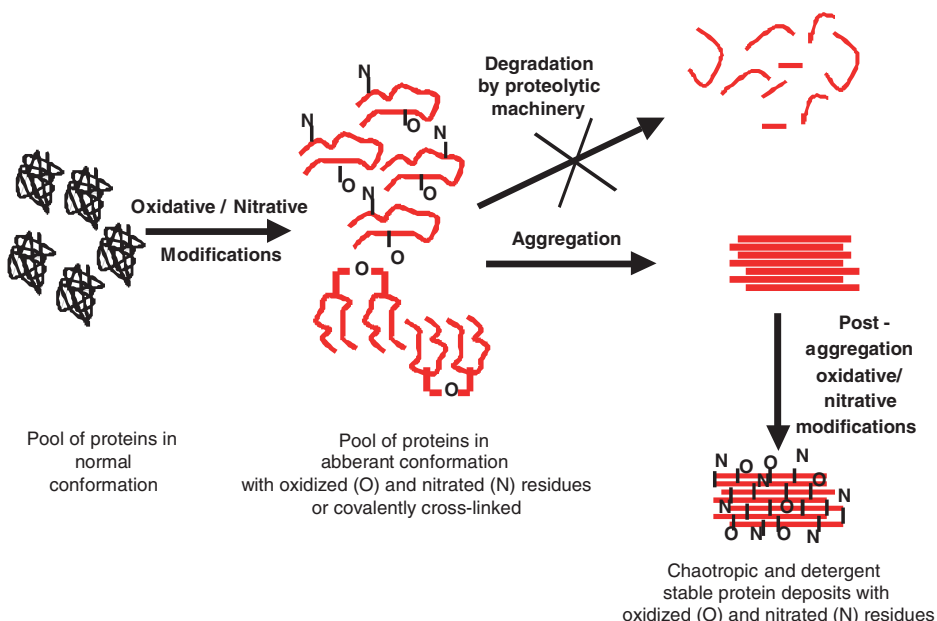


Figure 7-1. The role of oxidative and nitrative posttranslational modifications in initiation, propagation, or further stabilization of the aggregation process

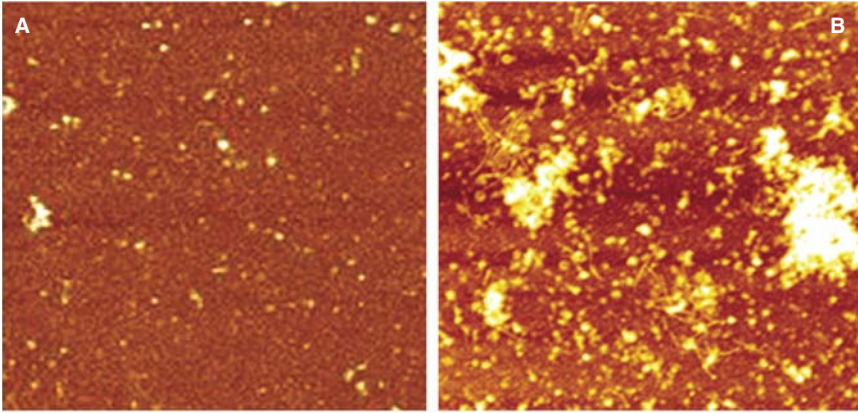


Figure 8.1-3. Hsp70/Hsp40 destabilize prefibrillar polyglutamine intermediates and facilitate the accumulation of higher order aggregates. (A) A $3\text{-}\mu\text{m}^2$ AFM image showing prefibrillar intermediates of an expanded huntingtin fragment, 1 hour after the initiation of aggregation. (B) A $3\text{-}\mu\text{m}^2$ AFM image showing that under identical conditions the presence of equimolar Hsp70/Hsp40 and an ATP-regenerating system decreases the detection of prefibrillar huntingtin intermediates and enhances fibril accumulation. (Adapted from Wacker *et al.*, 2004.)

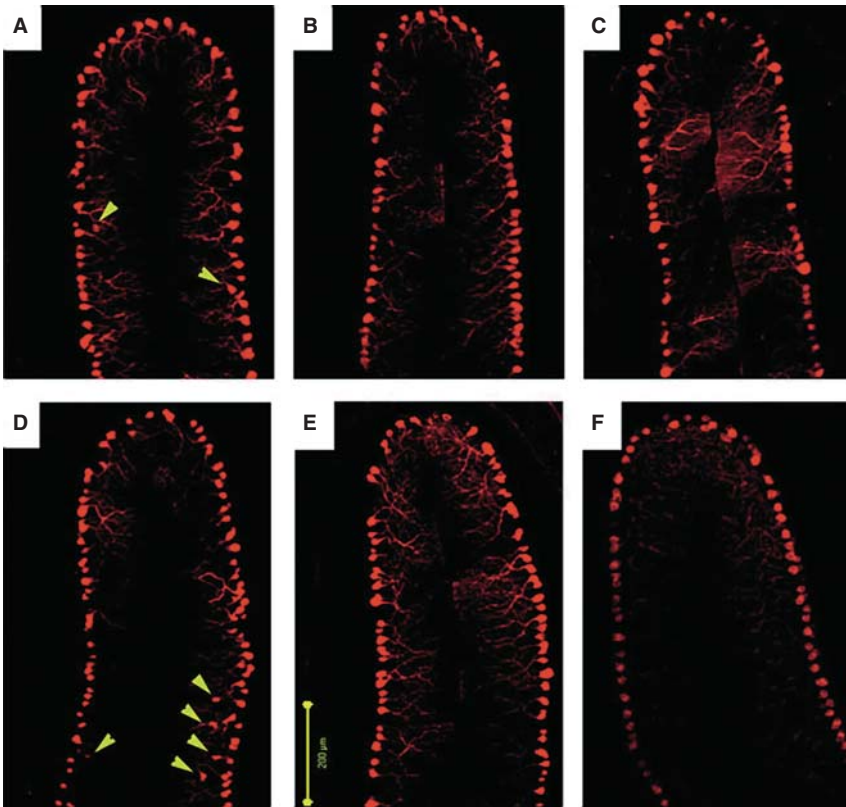


Figure 8.1-6. Overexpression of Hsp70 suppresses neuronal pathology in a SCA1 mouse model. (A,D) The progressive Purkinje cell morphology, including thinning of dendritic arbors and disruption of the Purkinje cell layer (arrows) in the BO5 SCA1 transgenic mouse at 9.5 weeks and 12 weeks, respectively. (B,E) Transgenic mice hemizygous for BO5 and Hsp70 show a mild improvement in Purkinje cell integrity at the same time points, whereas mice hemizygous for BO5 and homozygous for Hsp70 (C,F) showed a marked improvement in Purkinje cell morphology at both 9.5 and 12 weeks. (Reprinted with permission from Cummings *et al.* Copyright 2001 Oxford University Press.)

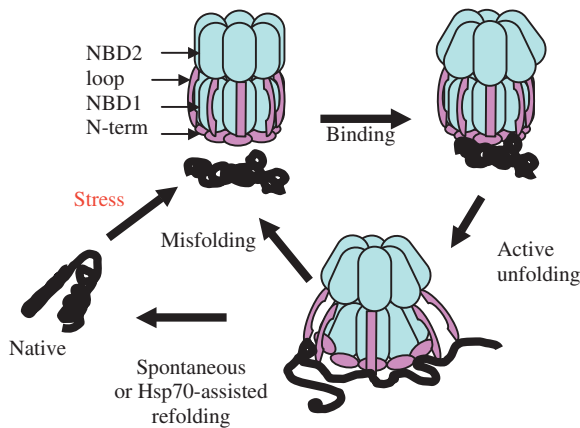


Figure 8.2-1. Proposed power-stroke mechanism for aggregate-unfolding by ClpB: Hexameric ClpB may interact with misfolded motives in aggregated proteins via its N-terminal domains and the loops protruding in between two conserved nucleotide-binding (NBD) domains. In the relaxed state, the ClpB₆ ring binds the substrate at several sites. ATP binding would then induce structural rearrangements in the ring, increasing the distance between binding sites and causing unfolding in the bound misfolded substrate. ATP-hydrolysis would allow dissociation and the spontaneous refolding of the disentangled aggregate

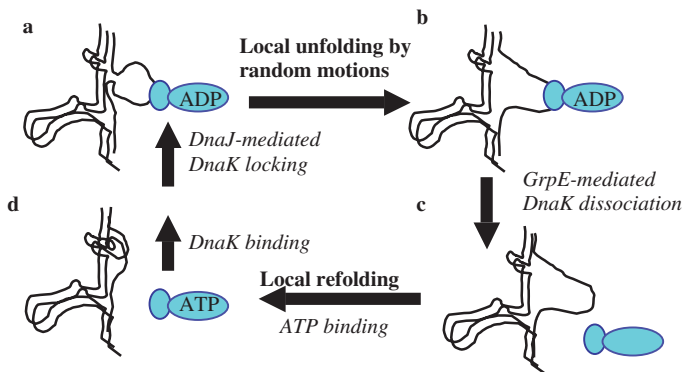


Figure 8.2-2. Suggested mechanism for DnaK-mediated unfolding of stable aggregates: (a) DnaK binds exposed hydrophobic loops flanked by collapsed misfolded segments in the aggregate. DnaJ induces ATP-hydrolysis that locks DnaK onto the loop. (b) Random motions of the locked DnaK in the ADP state locally unfold the misfolded segments flanking the loop. (c) GrpE-mediated ADP-release unlocks the chaperone. (d) The newly enlarged unfolded region can spontaneously refold into a native domain. Reiteration of the cycle at, or near the same site occurs until the whole protein has gradually reached its native conformation

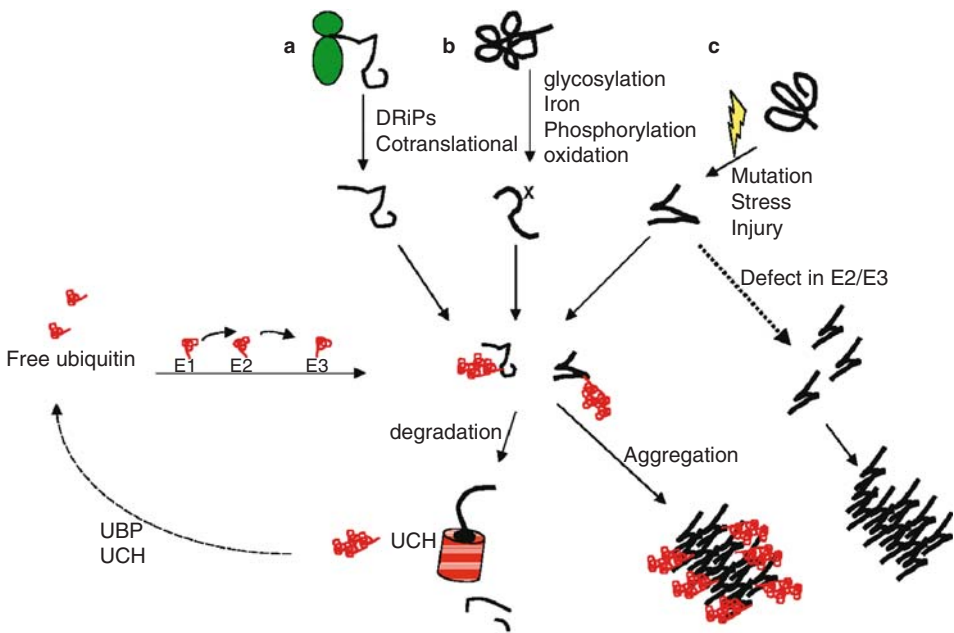


Figure 9-1. The Ub–proteasome pathway, protein degradation, and protein aggregation. The Ub–proteasome pathway of protein degradation is an enzymatic cascade that begins with the activation of free ubiquitin by an E1. This activated ubiquitin is then transferred to an E2, which can then interact with an E3 protein that will transfer the ubiquitin to a substrate (some classes of E3s will transfer the ubiquitin to itself prior to transfer to a substrate, and some E3s will serve as a platform to bring together a substrate and a charged E2 for ubiquitination). After a substrate has been targeted for degradation by multiple rounds of ubiquitination, its fate is sealed to one of two directions: degradation by the proteasome, or aggregation by association of hydrophobic domains. Degradation by the proteasome requires two more postubiquitination steps (the order of these two steps is not completely clear): (1) the substrate must be unfolded to enter the channel of the proteasome leading to the central catalytic chamber; (2) the substrate is deubiquitinated prior to entry into the catalytic chamber. Failure to execute either of these activities is sufficient to prevent proteasomal destruction of the protein and accumulation of ubiquitinated species. Mechanisms for generation of proteasome substrates. (A) Defective ribosomal products (DRiPs) and/or cotranslational degradation: recently translated mRNAs or mRNAs that are still being translated can be directed to the Ub–proteasome pathway. In this case, cellular conditions or specific defective transcripts direct the delivery of ribosomal products directly for destruction. Although at first this process appears to be quite a waste of cellular energy, the ability of cells to respond rapidly to changing environmental conditions is a positive adaptation to prevent inappropriate cellular chemistry. (B) Normal regulation of protein life-cycle: posttranslational modifications play a significant role in the generation of recognition motifs for E3 proteins to appropriate substrates. Specific E3s recognize glycosylation, iron, oxidation, and phosphorylation (see text for details). (C) Damaged proteins: cellular stress, genetic mutations, or injury can damage normal proteins creating recognition motifs for specific E3s. Cellular damage can also result in the inactivation of E2/E3 function compounding the cellular stress and accumulation of damaged, misfolded, and inactive protein. Inactivation of E2/E3 will prevent targeting to the proteasome and nonubiquitinated protein aggregates can accumulate

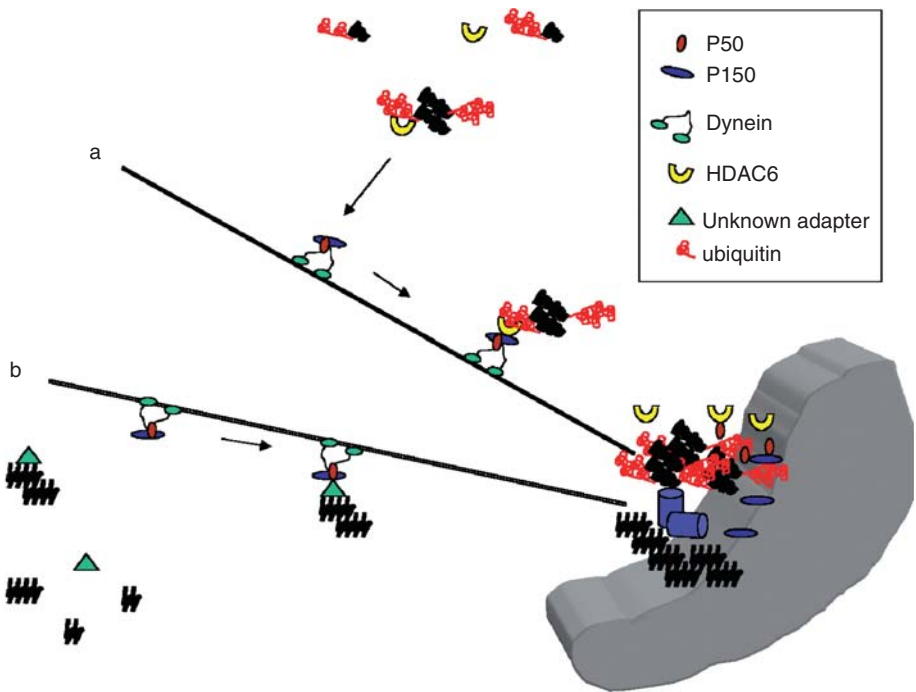


Figure 9-2. Molecular mechanisms of aggresome formation. Aggresome formation results from the accumulation of misfolded protein aggregates in a pericentrosomal location. The coalescence of these aggregates at one cellular location is an active process, requiring an intact MT network as well as specific microtubule motor proteins. Ubiquitinated microaggregates may be moved by different MT motors than nonubiquitinated aggregates. Some subunits of the motors become concentrated in aggresomes, but are not insoluble. See text for details. (A) Model for the movement of ubiquitinated microaggregates to aggresomes. Ubiquitinated microaggregates of a particular size are recognized through the Ub moieties by the histone deacetylase 6 (HDAC6) protein. HDAC6 also contains binding motifs for the dynein–dynactin MT motor complex. HDAC6 inhibitors, or siRNA for HDAC6, prevents the movement of ubiquitinated substrates to the aggresome, suggesting it is a molecular link for ubiquitinated, aggregated protein and the MT motor complex dynein–dynactin. (B) Proposed model for the movement of nonubiquitinated microaggregates of protein to aggresomes. Nonubiquitinated proteins have also been shown to form aggresomes in response to proteasome inhibition. The dynein–dynactin motor and MTs are required, however, the HDAC6 (see A) is not involved in this movement. Therefore, there may be yet another molecule that can identify nonubiquitinated aggregates of a particular size and link the aggregates to the dynein–dynactin motor. These molecules could be chaperones such as HSP90/HSP56 (FKBP52)/HSP70. The HSP 90-binding immunophilin FKBP52 (hsp56) has been shown to interact with steroid hormone receptors and cytoplasmic dynein, and this was suggested as a molecular mechanism for the trafficking of steroid nuclear hormone receptors through the cytoplasm to the nucleus. A similar mechanism may mediate the movement of nonubiquitinated protein aggregates. FK506, the immunosuppressant drug, does not disrupt the movement of Steroid hormone receptors on MTs, even though the PPI domain is required for FKBP52 binding to dynein, suggesting that the PPI activity is distinct from the PPI–dynein interaction surface.

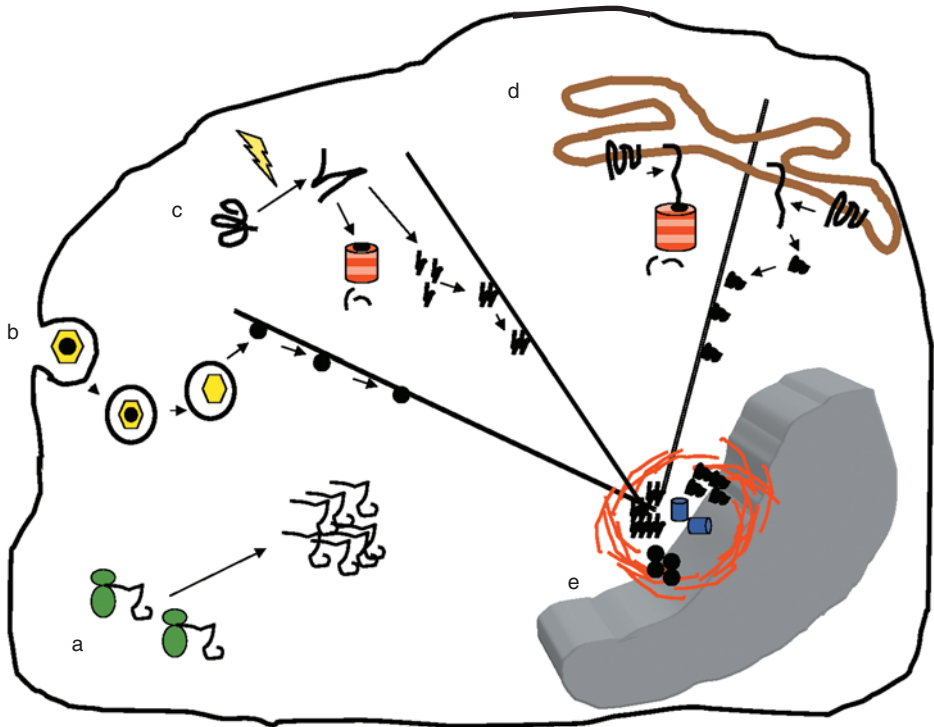


Figure 9-3. Aggresome formation in diverse cellular phenomenon. Since the description of aggresomes in 1998, a variety of mechanisms have been shown to result in the formation of aggresome structures. See text for details. (A) DALIS: in maturing DC, DRiPs are specifically concentrated and withheld from proteasomal degradation until the process of DC maturation is complete. After this time, the DRiPs are degraded by proteasomes and the resulting peptides are involved in MHC class I presentation of antigen to the immune system. The transient Ub-positive inclusions do not require MTs or rearrange vimentin. (B) Viral factories: viral infection of host cells by large DNA viruses begins by the uncoating of the virus in endosomes and the release of nucleoprotein particles into the cytoplasm. These particles then travel retrograde along MTs and concentrate at the centrosome where the later viral genes are then turned on and the virus begins the process of replication and repackaging. Viral factories require intact MTs and are accompanied by rearranged IFs. (C) Soluble cytoplasmic proteins: genetic mutations and/or cellular stress can result in misfolding of normally soluble cytoplasmic protein and thus be targeted to proteasomes for destruction. The inability of the proteasome to efficiently and quickly degrade substrates results in the formation of aggresomes that bear all of the standard hallmarks of aggresomes. (D) Membrane proteins and secretory proteins: genetic mutations, cellular stress, or simply inefficient folding processes of wild-type membrane and secretory proteins result in the retrotranslocation of recognized substrate from the ER back into the cytoplasm for degradation by proteasomes. Genetic mutations that decrease the efficiency of protein folding will increase the number of substrates to the proteasome. In the classic example of aggresomes, the 508 mutation in the CFTR protein prevents the mutant protein from transiting through the ER to the cell surface. Rather, the majority of 508CFTR is retrotranslocated from the ER to the cytoplasm and degraded by proteasomes. In cases where very high levels of 508CFTR are expressed it is possible to overload the capacity of the proteasome and the resulting cytoplasmic inclusions of highly ubiquitinated, insoluble 508CFTR form via the transit of small microaggregates of Ub-508CFTR protein along MTs to the centrosome, where they are surrounded by collapsed IFs. (E) Aggresome formed at the centrosome is surrounded by rearranged intermediate filaments, and displays a deformation of the nuclear envelope.

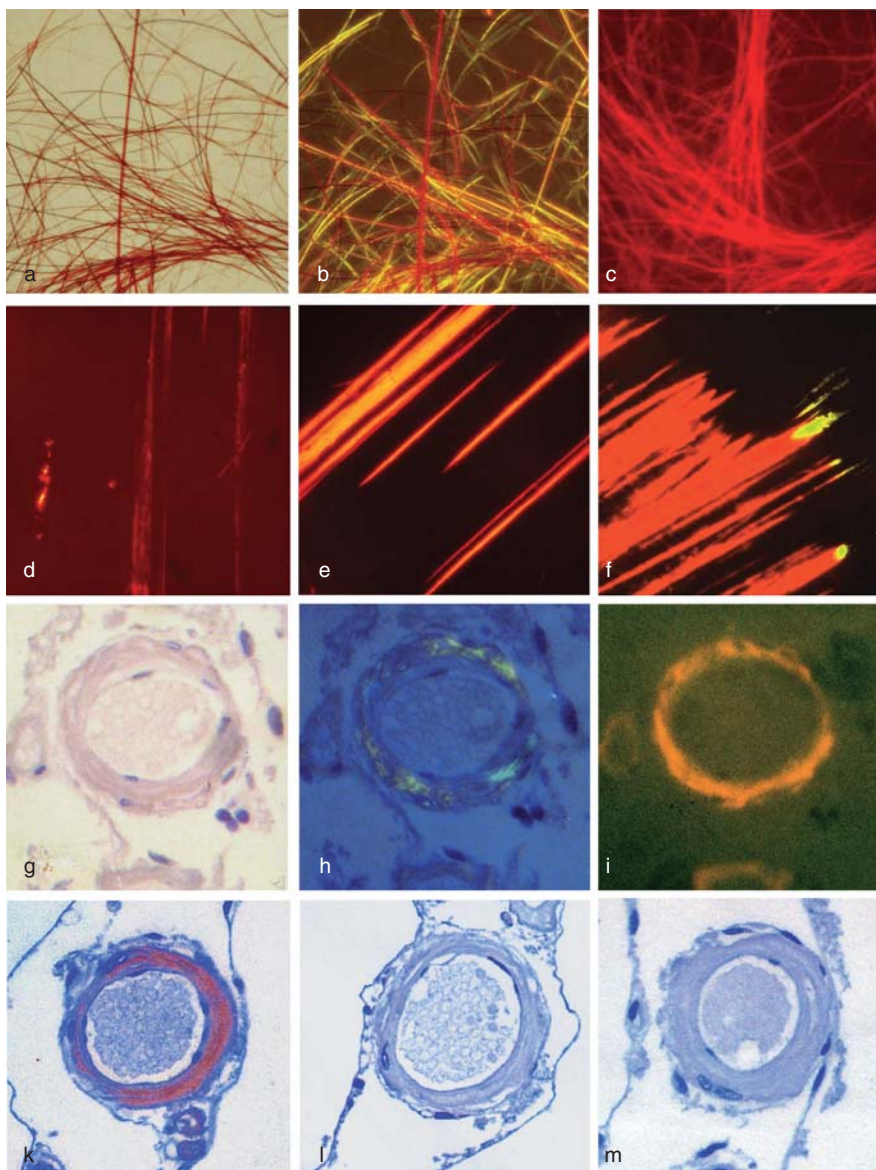
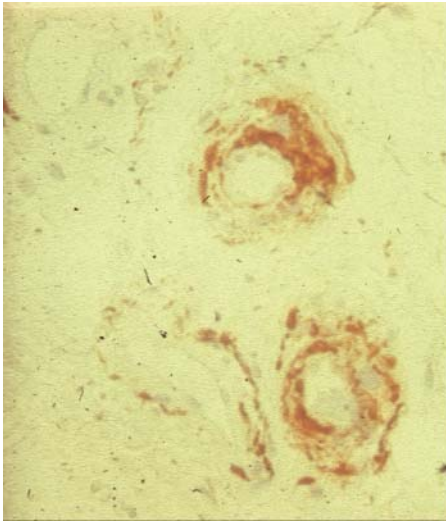
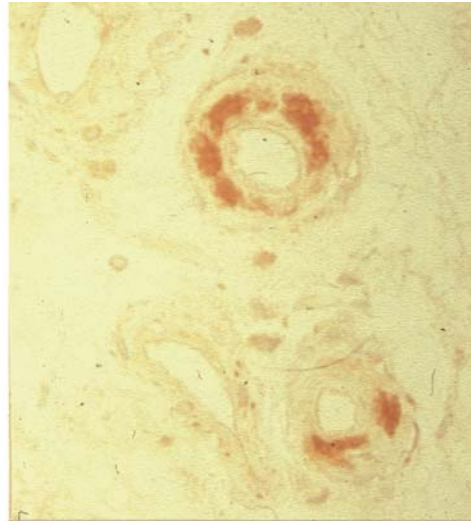


Figure 11.1-3. Optical properties of CR crystals (a–f) and the diagnosis of amyloidosis (g–m). (a) CR crystals of different diameters are dark red in bright light. (b) Dependent on their diameter, between crossed Nicols, CR crystals display differently colored anisotropy. (c) These crystals are uniformly bright fluorescent red (excitation in green with a red barrier filter). (d–f) CR crystals lacking orientation and anisotropy (black in polarized light) show anisotropy only when oriented by scratching. (a) Scratches in the “polarization shadow” (see Section 6.11) only some irregularities; (b) when the scratches are turned by the slide table, the very bright orange (thinner CR-film in midscratch) and red anisotropy at the rim (thicker CR film) is seen, but only where CR crystals are oriented by scratching. (f) With decreasing thickness, that is, where scratches are terminated, the anisotropy turns from intensively red to orange over yellow to green, displaying all the characteristics of CR crystals and CR stained amyloid with comparable thickness. (g–i) Tinctorial diagnosis of amyloid in tissue sections (Leptomeningeal amyloid angiopathy in Alzheimer’s disease): (a) CR staining of leptomeningeal vessel lacking the characteristic salmon-red coloration, (b) green birefringence appears between crossed Nicols, which proves the binding of CR and the presence of the amyloid. However, not all parts of the amyloid is illuminated, indicated by the presence of the “polarization shadow” (see Section 6.11); (i) CRF displays bright yellow-orange fluorescence of the entire amyloid and illuminates all amyloid present at the same time. (k–m) Immunohistochemistry identifies the clinical syndrome by revealing the chemical nature of the amyloid as A β -derived. Three examples (of the entire set of results covering all major amyloid syndromes) are shown: (k) anti-A β [positive, red coloration is congruent with the CRF in (i)]; (l) anti-APrion (negative); (m) anti-ATTR (negative)

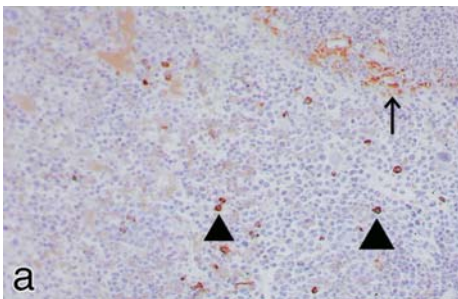


Anti-AA

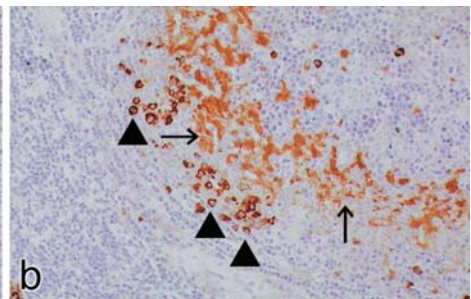


Anti-Aβ₂M

Figure 11.1-6. Two different amyloid classes in one patient. Artery of a rectal biopsy (see Section 7.4)

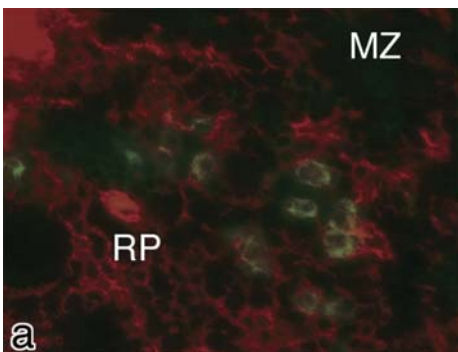


a

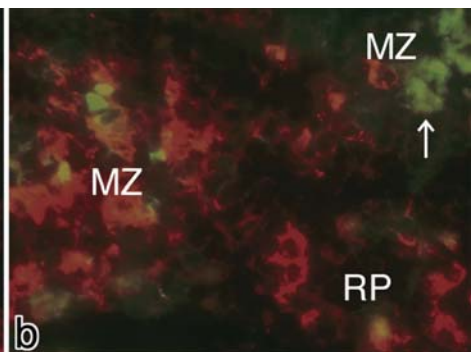


b

Figure 11.2-1. Immunolocalizations of F4/80 positive macrophages (arrowheads) on days 7 (a) and 14 (b). The macrophages increased near the amyloid deposition (arrows). $\times 120$



a



b

Figure 11.2-2. Immunolocalizations of F4/80 (red) and CSPG (green) on day 3 (a) and 14 (b). Yellow colors are overlaid by two labeling patterns (red and green). MZ, marginal zone; RP, red pulp. Arrows, Amyloid deposition. $\times 300$

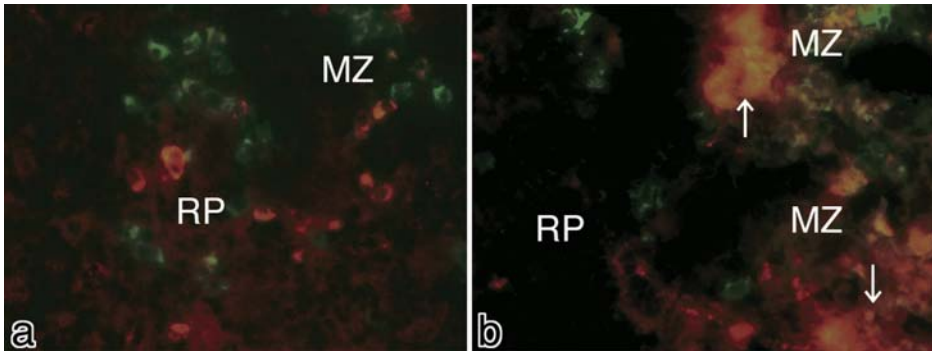


Figure 11.2-3. Immunolocalizations of APC (red) and CSPG (green) on day 3 (a) and 14 (b). Yellow colors are overlaid by two labeling patterns (red and green). MZ, marginal zone; RP, red pulp. Arrows, Amyloid deposition. ×300

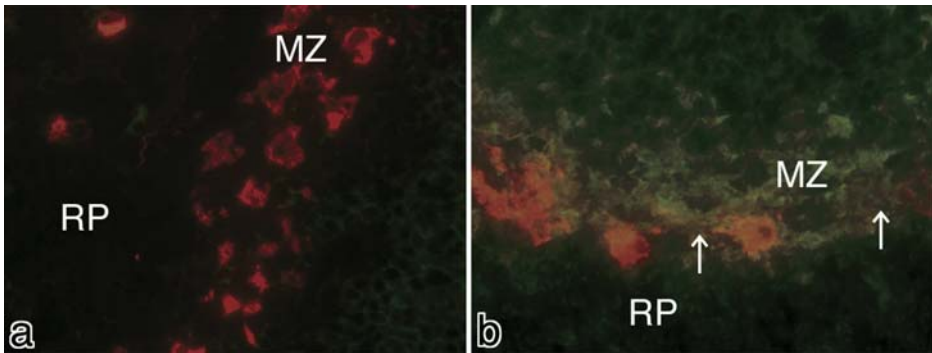


Figure 11.2-4. Immunolocalizations of ER-TR9 positive macrophages (red) and CSPG (green) on day 3 (a) and 7 (b). Yellow colors are overlaid by two labeling patterns (red and green). MZ, marginal zone; RP, red pulp. Arrows, Amyloid deposition. ×300

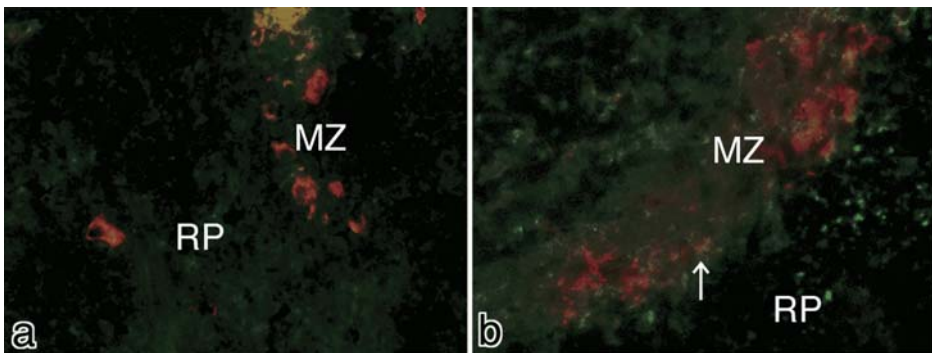


Figure 11.2-5. Immunolocalizations of ER-TR9-positive macrophages (red) and APC (green) on days 3 (a) and 7 (b). Yellow colors are overlaid by two labeling patterns (red and green). MZ, marginal zone; RP, red pulp. Arrows Amyloid deposition. ×300

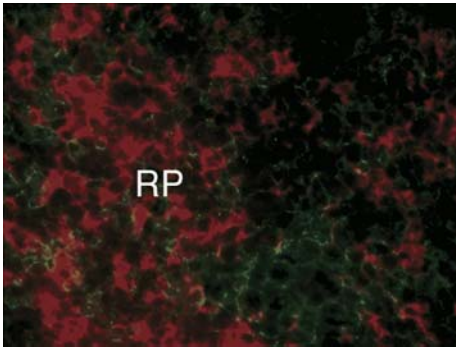


Figure 11.2-6. Immunolocalizations of F4/80 positive macrophages (red) and APC (green) on day 3. MZ, marginal zone; RP, red pulp. $\times 300$

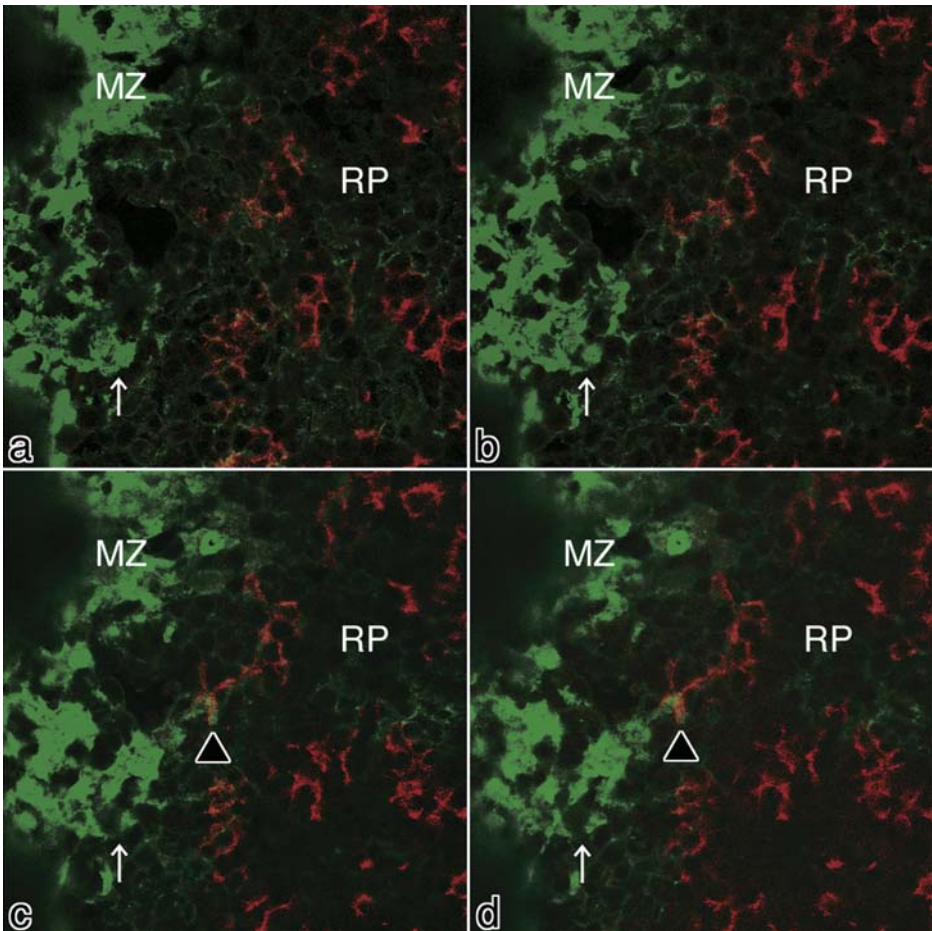


Figure 11.2-7. Series of four sections (a–d) taken by confocal scanning laser microscopy. Splenic amyloid deposition is stained with anti-F4/80 positive macrophages (red) and anti-amyloid P component (green) on day 14. The immunolocalization of APC and F4/80-positive macrophages are observed as separate positions (a and b). However, the F4/80-positive macrophages near the amyloid deposition are connected and closely related (arrow heads) with the APC in the deeper sections (c and d). The images are captured at intervals of approximately $1.5\mu\text{m}$. Yellow colors are overlaid by two labeling patterns (red and green). MZ, marginal zone; RP, red pulp. Arrows, Amyloid deposition. $\times 425$

Figure 11.2-8. Electron micrograph of splenic amyloidosis shows the fibroblasts in large amounts of amyloid fibrils in the extracellular area of the marginal zone on day 7 (b). Amyloid fibrils are seen in the extracellular distribution from the cytoplasm of the fibroblasts. (a) A higher power view of arrow head in (b). Amyloid fibrils extend from the cytoplasm of the fibroblasts into the extracellular distribution. Arrows, Amyloid fibrils. (a) $\times 20,000$. (b) $\times 7200$

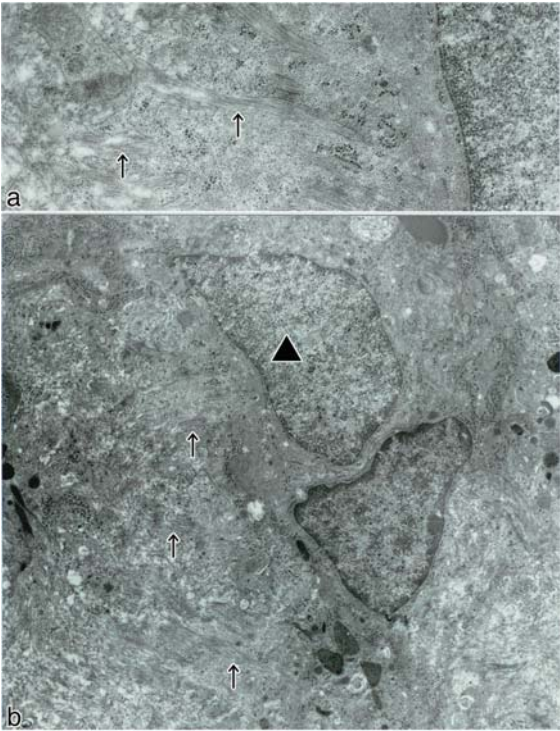
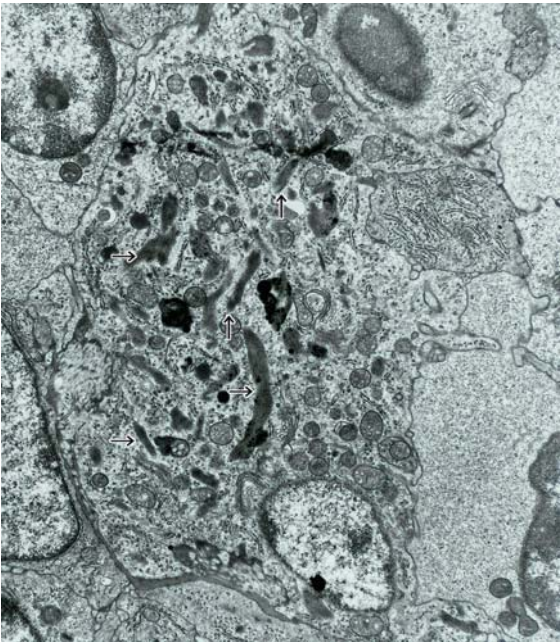


Figure 11.2-9. Electron micrograph of macrophage in the amyloid deposition area on day 14. The macrophage contains lysosomal-derived fibrillar structures of amyloid fibrils (arrows). There were many rough endoplasmic reticula and Golgi apparatuses in the macrophages. $\times 7600$



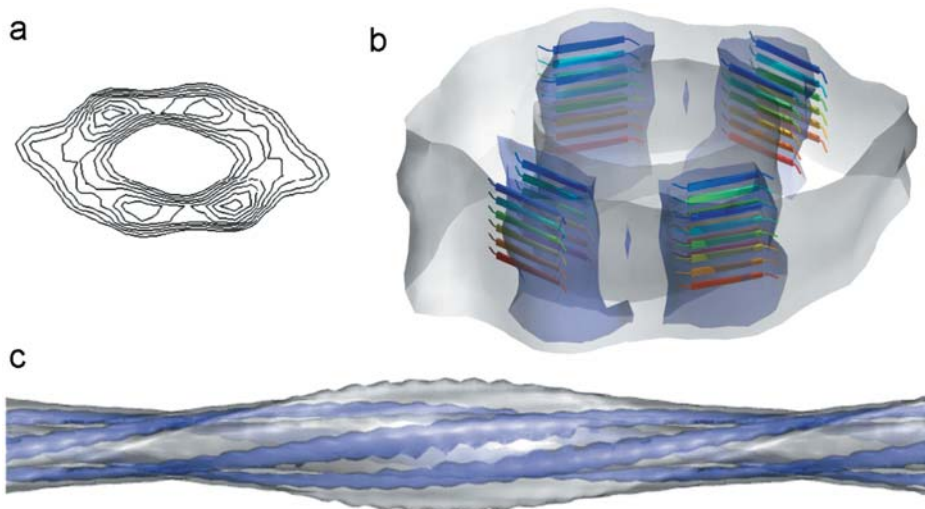


Figure 12.2-5. 3D structure and cross- β model of SH3 fibrils. (a) Contour plot of a cross-section through the 3D density map of an SH3 fibril. Four regions of high density correspond to protofilaments, and some weaker density is attributed to loops. The four protofilaments surround a hollow core with a maximum diameter around 50 Å. (b) Segment of the 3D structure with a generic cross- β model of the protofilament structure. Two contour levels are used to show the map as transparent grey and blue surfaces. The fibril axis runs near the vertical direction, with a slight tilt towards the viewer. (c) Rendered view of a longer segment of the fibril, with the fibril axis horizontal. The higher density contour shown in blue highlights the protofilaments, and it is enclosed by the lower density contour in grey.

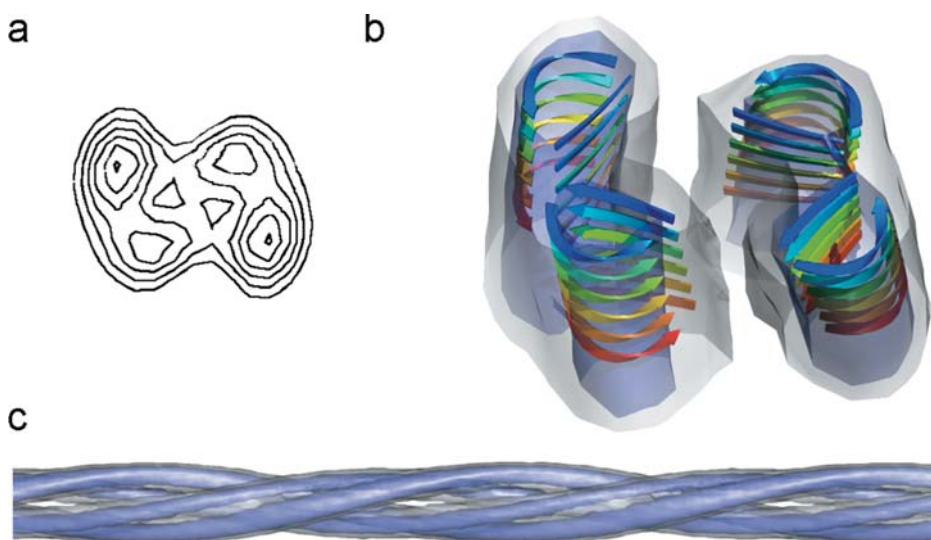


Figure 12.2-6. 3D structure and cross- β model for insulin fibrils. (a) contour plot, (b) 3D structure with density iso-surfaces shown in grey and blue, with a fitted cross- β model, and (c) fibril view as for Figure 7-5. In this case, there are also four protofilaments, but little other density, and the packing is more compact. The cross- β model shows a curved and a flat sheet in a parallel arrangement, to take account of the three disulfide bonds present between and within chains in the insulin molecules forming these amyloid fibrils. (From Jiménez *et al.*, 2002.)

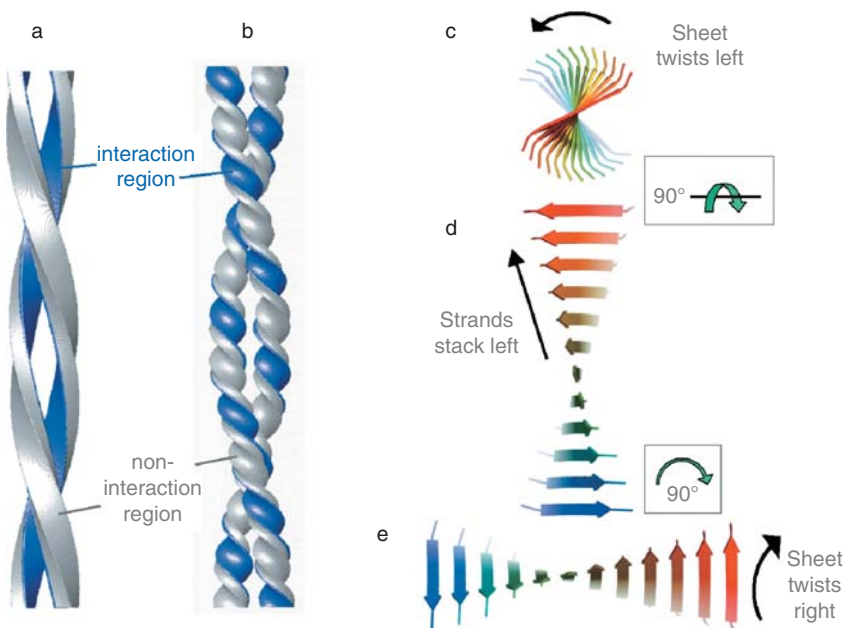


Figure 12.2-7. Schematic diagrams explaining the relationship between β -sheet and protofilament twist. (a) A fibril formed of two protofilaments, shown as ribbons, twisting around each other and keeping the same surface (colored) in contact. (b) Two highly twisted protofilaments coiling around each other, showing that the contact surface is not maintained if the twist of each protofilament does not match the overall coiling of the fibril. (c) The natural twist of β -sheets in proteins is left-handed, as seen by viewing the sheet edge-on. The receding strands twist counterclockwise. A parallel β -sheet is shown for clarity. (d) If the structure is turned through 90° as shown by the green arrow, the sheet still appears left-handed (the strands recede towards the left). (e) However, if the view is rotated by 90° in the plane of the page, the sheet appears right-handed (the direction of rotation is clockwise in this view). The left-handed twist shown in (d) must match the overall left-handed twist of amyloid fibrils to form structures with conserved contacts as shown in (a). (a) and (b) are adapted from Jiménez *et al.* (2002). Copyright 2002 from the National Academy of Sciences USA

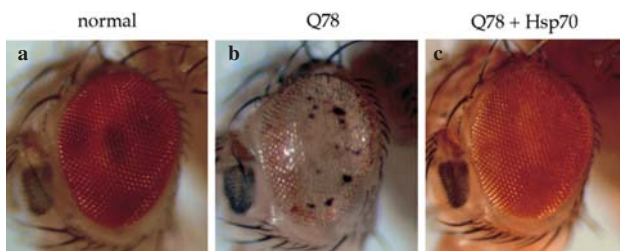


Figure 13.1-2. Examples of the phenotypes of various *Drosophila* models of human neurodegenerative diseases. (a) The eye of a wild-type fly. (b) Eye of a fly expressing a pathogenic form of the human ataxin-3 protein (SCA3tr-Q78) shows severe degeneration, including loss of external eye pigmentation. (c) Coexpression of Hsp70 strikingly suppresses the SCA3-trQ78 neurodegenerative phenotype

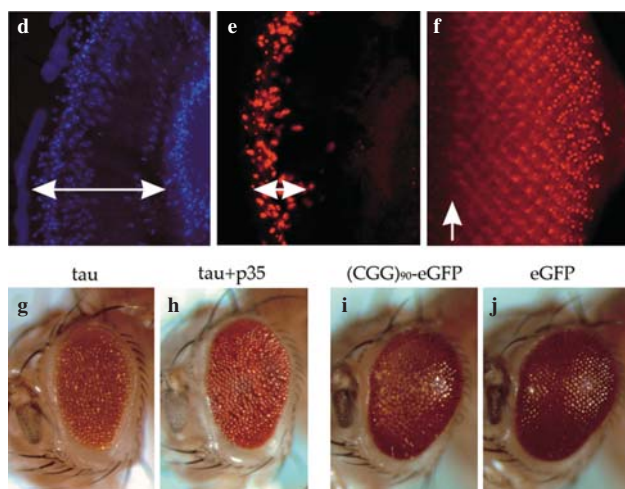


Figure 13.1-2. (d) Internal section of a normal adult fly eye stained with Hoescht shows the regular nuclear distribution in the retina. (e) Internal section of an eye from a fly expressing pathogenic SCA3tr-Q78, shows a severely collapsed and thin retinal layer, with striking accumulation of the protein in inclusions (red, HA immunostaining). (f) Larval eye imaginal disc of an animal expressing SCA3tr-Q78 shows the onset of aggregate formation early in development. Reference for these flies and phenotypes are Warrick *et al.* (1998, 1999). (g) Wild-type tau induces degeneration in the eye, causing a rough and reduced eye phenotype. (h) Coexpression of the anticell death baculoviral protein p35 has a mild suppression effect on tau-induced degeneration. References for these flies and phenotypes are Wittman *et al.* (2001) and Jackson *et al.* (2002). (i) Expression of an fmr-1-related expanded repeat (CGG)₉₀ in the noncoding 5' UTR of a marker gene eGFP induces retinal degeneration. (j) Similar expression of eGFP alone. Reference for these flies and phenotypes is Jin *et al.* (2003). Flies of genotype: *gmr-gal4/UAS-SCA3tr-Q78* (b, e, f); *gmr-gal4 UAS-SCA3tr-Q78/UAS-hHsp70* (c); *gmr-gal4/UAS-tau* in (g); *gmr-gal4 UAS-tau / UAS-p35* in (h); *gmr-gal4/ UAS-(CGG)₉₀-eGFP* in (i); *gmr-gal4/UAS-eGFP* in (j)

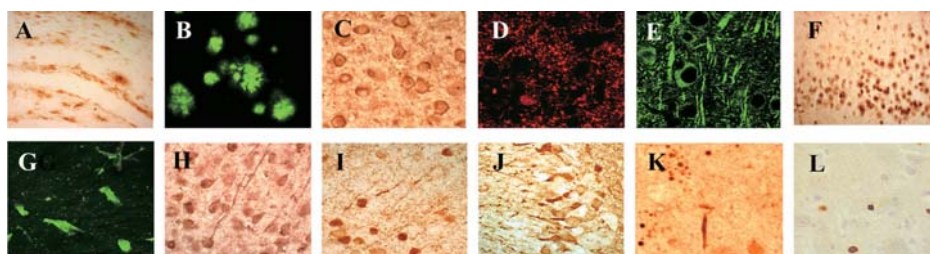


Figure 13.2-2. Neuropathological aspects of genetically engineered models of neurodegeneration. (A) Diffuse amyloid plaques in PDGFβ-hAPP Sw tg mice. (B) Mature neuritic plaques in the frontal cortex of mThy-1-hAPP751 mutant tg mice. (C) Tau hyperphosphorylation in the hippocampal neurons of the PDGFβ-hAPP Sw tg mice. (D) Synaptic alterations visualized with the confocal microscope in sections immunostained with an antibody against synaptophysin in mThy-1-hAPP751 mutant tg mice. (E) Dendritic alterations visualized with the confocal microscope in sections immunostained with an antibody against MAP2 in mThy-1-hAPP751 mutant tg mice. (F) Neuronal alterations visualized with the confocal microscope in sections immunostained with an antibody against NeuN in hAPP × sCrry tg mice. (G) Amyloid angiopathy in hAPP × TGFβ1 tg mice. (H) Tangle-like neurofibrillary pathology in the hippocampus of mThy-1-Tau P301L tg mice. (I, J) Lewy body-like inclusions and Lewy neurites immunostained with an antibody against α-synuclein in PDGFβ-α-synuclein tg mice. (K) SOD1 inclusions in tg mice. (L) Ubiquitin-immunoreactive intranuclear inclusions in HD tg mice

The Aggresome: Proteasomes, Inclusion Bodies, and Protein Aggregation

Jennifer A. Johnston

1. Abstract

The accumulation of misfolded protein into insoluble inclusions is a pathological hallmark of many diseases. How these inclusions form and their role in the degenerative process is still unknown. Recently, a cellular response to the accumulation of misfolded protein was described, and the resulting structures were termed Aggresomes. Aggresomes occur in cells due to impairment of intracellular degradation pathways, and are insoluble inclusions associated with a rearranged intermediate filament network. Aggresomes form by the microtubule and dynein–dynactin-dependent delivery of small microaggregates of protein to a central cellular location, in most cases the centrosome. In this chapter, the characteristics of aggresomes are described, followed by a discussion of the relationship of aggresomes to the general class of ubiquitin-intermediate filament diseases (most notably characterized by Lewy Bodies in Parkinson’s Disease, intranuclear inclusions in Huntington’s Disease, and Bunina Bodies in amyotrophic lateral sclerosis), and conclude with potential mechanisms for aggresome formation. Aggresomes have the potential to provide a mechanistic clue to the pathogenesis of ubiquitin-intermediate filament disorders.

2. Introduction

The evolution of multicellular organisms is consistent with the notion that adaptive functions will be beneficial and maintained, especially if those functions offer a benefit to combat inherent rates of error due to the nature of existence. Every eukaryotic cell is constantly involved in the production and delivery of new protein to sustain normal cellular functions. The ability to appropriately regulate and control the delivery of perfectly formed protein at precisely the right cellular time is a remarkable achievement in cellular selection processes. This feat is achieved in part by the chaperone systems in cells that serve to guide a protein along its folding pathway until it has achieved a “final” folded state (Fink, 1999; Kregel, 2002), and in part by the protein degradation mechanisms of the cell (Ciechanover, 1998). In the case that the cell’s chaperone machinery and degradation machinery cannot cope with a particular protein or cellular condition, cells have evolved a manner in which to prevent these malformed proteins from impairing cellular function—at least for a while. In 1998, a pathway was discovered that allows a cell to continue to function, even when the amount of protein mistakes, errors, and hopelessly misfolded protein overwhelm the aid given by molecular chaperones/degradation. The end result of this pathway was called an Aggresome, and the pathway that resulted in their formation was ascribed to the accumulation of misfolded protein in cells (Johnston *et al.*, 1998).

Protein misfolding can arise from intracellular damage or stress, or from genetic mutations. In general, intracellular degradation pathways recognize malformed and damaged proteins and eliminate them as part of a general cellular housekeeping mechanism. Quality control surveillance exists throughout the life of a protein, from the time of emergence from the ribosome, until the end of functional purpose. Our current understanding of protein folding and quality control suggests that the chaperone proteins [also known as heat-shock proteins (HSPs)], from their initial discovery as heat-induced proteins) fulfill a major role in helping a protein achieve its “final” folded state. HSPs associate with nascent proteins in the cytoplasm (Kregel, 2002), Bip, calnexin, GRP94, and calreticulin assist protein folding in the endoplasmic reticulum (ER) (Kaufman, 1999), and still other HSPs associate with proteins that are struggling to form a functional, energetically favorable conformation (Lindquist and Kim, 1996; Glover and Lindquist, 1998). Some HSPs are also involved in intracellular trafficking mechanisms (HSP56/FKBP52 and HSP90) (Silverstein *et al.*, 1999; Galigniana *et al.*, 2001). These assistant pathways are cytoplasmic as well as in the secretory pathway, and ensure that only the functional proteins are involved in crucial cellular activities. The main pathway for the elimination of nonfunctional, damaged, or aged proteins is through the ubiquitin (Ub)–proteasome pathway of protein degradation, and this pathway also operates on cytoplasmic (Ciechanover, 1998) as well as secretory proteins (Kostova and Wolf, 2003). The accumulation of misfolded protein associated with pathological conditions is thought to occur either due to a problem in protein folding that renders the protein inaccessible to cytoplasmic proteasome proteolysis (e.g., genetic mutation or modification), or because of a defect in the proteolytic capacity of the cell.

In this chapter we will discuss the characteristics of aggresomes, and how this pathway defines a cellular response to the presence of misfolded proteins. This pathway suggests a mechanism for the concentration of misfolded monomers/multimers in a cellular microdomain that could generate the energetically favorable formation of fibrils. A continuing mystery, even with our knowledge of aggresome formation, is whether fibrils (or the fibril-forming process) are injurious for cells or serve a protective mechanism that allows normal cellular function to remain intact, even while misfolded protein accumulates. First, a description of the known characteristics of aggresomes is provided, followed by a discussion of the data linking aggresomes to various pathological conditions. The third section of the review will discuss potential mechanisms for aggresome formation, particularly in neurons, and finally, the fourth section will conclude with a perspective for future directions.

3. Characteristics of Aggresomes

Aggresomes are intracellular accumulations of misfolded, aggregated protein. Aggresome formation occurs when the capacity of the Ub–proteasome pathway has been overwhelmed (Johnston *et al.*, 1998). Aggresomes are generally Ub positive, but can also be Ub negative (Garcia-Mata *et al.*, 1999). The formation of aggresomes is a microtubule (MT)-dependent process, whereby small microaggregates of protein are transported along MTs by a dynein–dynactin motor (Garcia-Mata *et al.*, 1999; Johnston *et al.*, 1998, 2002). In cultured dividing cells, the microaggregates accumulate at the centrosome, the terminus of the minus end of the MT. The accumulation of aggregated protein at the centrosome is accompanied by a rearrangement of the intermediate filament (IF) protein vimentin, such that the vimentin collapses to form a cage around the aggresome (Johnston *et al.*, 1998). In this section, we will discuss in detail the various characteristics of aggresomes, and their implications for understanding integrated cellular pathways and microdomains.

3.1. Aggresomes Are Composed of Aggregated, Undegraded Protein

The majority of cellular protein degradation occurs through the action of the Ub–proteasome pathway (Rock *et al.*, 1994, and **Figure 9-1; see color insert**). Regulatory proteins, such as those involved in cell cycle (Koepp *et al.*, 1999), transcription regulation (Conaway *et al.*, 2002), and apoptosis (Jessenberger and Jentsch, 2002), rely on a highly coordinated series of events to appropriately recognize and remove protein activity. The proteasome plays a central role in antigen presentation (Michalek *et al.*, 1993; Rock and Goldberg, 1999) by MHC class I. In addition, proteins with nonmethionine N-termini (Varshavsky, 1997) and damaged proteins (Tsirigotis *et al.*, 2001) are removed by the Ub–proteasome pathway. Because the specific mechanistic details of the components of the Ub–proteasome pathway could fill several libraries, the reader is directed to a few important references for additional information from that given in this review (Ciechanover, 1998; Hershko and Ciechanover, 1998; Hershko *et al.*, 2000; Sherman and Goldberg, 2001), and specifically for the Ub pathway in neurons, see Johnston and Madura (2004). The complete proteolysis of a protein to its constituent amino acids offers an immutable finality to its biochemical activity. This incontrovertible end is achieved though the action of the multiple catalytic activities of the 26s proteasome. The proteasome is a 26s complex of proteins that form from the assembly of a 20s core cylinder with a 19s cap (reviewed in Lupas *et al.*, 1993; Zwickl *et al.*, 2001). The proteolytic active sites of the proteasome are contained within the central internal chamber (Kruger *et al.*, 2001) of the 20s particle, and the 19s cap contains a variety of subunits that bind ubiquitinated protein (van Nocker *et al.*, 1996), remove Ub and hold the 19s cap to the 20s core (Leggett *et al.*, 2002), and possess ATPase activity (Rubin *et al.*, 1997; Braun *et al.*, 1999; Benaroudj *et al.*, 2003). The 20s core proteasome particle is able to degrade nonubiquitinated, unfolded protein (Wenzel and Baumeister, 1995). The 26s proteasome has essentially the same 20s core cylinder; however, the addition of the 19s cap serves to carefully regulate the opening of the central cylinder for entry of Ub-targeted substrate (Groll *et al.*, 2000). The proteasome is able to degrade many diverse proteins through the action of three distinct protease activities in the central chamber (Zwickl *et al.*, 2001), and the action of these proteases is suggested to aid in the directional movement of substrate through the catalytic channel (cylinder) (Kisselev *et al.*, 1999). The product of the protein processing through the proteasome is the generation of small peptides that can be acted upon by cytoplasmic proteases to generate free amino acids or can be transported in to the ER for MHC class I loading and immune-related functions (reviewed in Kloetzel, 2001). Proteasome substrates are channeled to the mouth of the proteasome by the sequential action of a group of enzymes, creatively named E1, E2, and E3, that function to add the 76 amino acid protein Ub to substrates as a tag for degradation. E1, E2, and E3s are also involved in the regulated endocytosis mechanisms that lead to the delivery of plasma membrane transmembrane protein to lysosomes (Hicke and Dunn, 2003). The distinction between these two degradation pathways exists in the Ub signal that is added to a protein. Proteasome degradation requires the addition of multiple Ub moieties onto a substrate (Pickart, 2000). A Ub chain (also referred to as a “ladder” on SDS-PAGE) that contains four or more Ub moieties will form a hydrophobic patch that has affinity for particular proteasome subunits (Deveraux *et al.*, 1994; Sloper-Mould *et al.*, 2001; Lam *et al.*, 2002). Endocytosis and delivery to lysosomes requires the addition of one Ub moiety, and this monoubiquitin then allows interaction with endocytosis adapter proteins like epsins to facilitate endocytosis (Hicke and Dunn, 2003). In our discussion of the formation of aggresomes, a focus on the degradation by proteasomes is emphasized. However, in the Section III, Mechanisms of Aggresome Formation, it will be important to reconsider the involvement of the monoubiquitination/endocytosis pathway in cellular dysfunction.

How do substrates become selected for elimination? As outlined in **Figure 9-1; see color insert**, there are many ways that a protein can be recognized for permanent removal via destruction

by proteasomes. All of these independent mechanisms for the generation of proteasome substrates begin with the activation of free Ub by Ub-activating enzymes (UBA), or E1s. There are only two known E1s, and they function in the nucleus or cytoplasm (Stephen *et al.*, 1996). After Ub has been activated through thio-ester linkage between E1 and the C-terminus of Ub, the charged Ub is transferred to the active site cysteines of a Ub conjugating enzyme (UBC), or E2. The E2s are a larger class of enzymes than the E1s and contain about 20 members (Jones *et al.*, 2002). E2s carrying their charged Ub moiety then interact with an E3, or Ub ligase (Lorick *et al.*, 1999). E3s function to recognize substrate and aid in the transfer of Ub from the E2 to formation of an isopeptide bond between the C-terminus of Ub and the ϵ -amino group of a lysine in the substrate. There are at present estimated to be 600 E3 ligases in the human genome, and genomic research is likely to expand the numbers of E1, E2, and E3 enzymes identified (Semple *et al.*, 2003). However, the functional diversity of the Ub pathway suggests that there will always be greatest diversity at the level of substrate recognition by E3s. Many Ub ligases are enormous protein complexes, composed of many subunits (Deshaies, 1999; Maniatis, 1999; Joazeiro and Weissman, 2000; Hatakeyama and Nakayama, 2003; Fuchs *et al.*, 2004; Ingham *et al.*, 2004). These subunits are rarely static members, and there are numerous examples of ligases that change constituencies throughout the cell cycle (Spruck and Strohmaier, 2002). The significance of this massive diversity at the level of the ligases is to create an amazing and highly specific diversity in the ability to recognize degradation motifs. For example, in the Skp-cullin-Fbox (SCF) complex ligase (Deshaies, 1999), the Fbox subunit serves to recognize specific motifs. In muscle, the FBX4 protein subunit in the SCF ligase functions to recognize specific phospho-epitopes of proteins and does not recognize nonphospho forms (den Engelsman *et al.*, 2003). Alternatively, the FBX2 of SCF ligase specifically recognizes sugar chains on cytoplasmic proteins (Yoshida *et al.*, 2002; Mizushima *et al.*, 2004). The Von Hippel Lindau ligase recognizes the removal of a hydroxylated proline in the hypoxia inducible factor (HIF) transcription factor protein (Jaakkola *et al.*, 2001). The hydroxylation of this protein thus serves as a sensor for the redox state of the cytoplasm, and cellular changes that lead to a loss of this hydroxylation lead to a loss of the protein (via degradation) and a change in transcription. The Nedd4 ligase serves to aid in endocytic pathways (Dinudom *et al.*, 1998). Undoubtedly, future work on E3 ligases will reveal many elegant examples of molecular sensors that are turned off by their destruction by proteasomes. There are also E3 ligases such as HSP70-ChiP that collaborate with cellular chaperones to recognize misfolded proteins and lead them to degradation (Demand *et al.*, 2001; Murata *et al.*, 2001; Wiederkehr *et al.*, 2002). These ligases are part of the cellular protein triage that maintains a constant surveillance in the cells to ensure that misfolded proteins are aided and refolded, or removed, rapidly.

After a substrate has been recognized by an E3, and multiple rounds of E2/E3 Ub conjugation have added multiple Ub to a substrate, the result is the formation of a multi-Ub chain that adopts a specific 3D structure (Sloper-Mould *et al.*, 2001). The multi-Ub chain has affinity for subunits of the proteasome cap (Deveraux *et al.*, 1994; van Nocker *et al.*, 1996; Thrower *et al.*, 2000), and this interaction is believed to dock the substrate at the mouth of the proteasome. Prior to entering into the catalytic channel, a substrate must be unfolded (Johnston *et al.*, 1995). There are about six known ATPases that reside in the proteasome cap, and these proteins serve to unfold substrate before it can enter into the channel (Navon and Goldberg, 2001; Benaroudj *et al.*, 2003). Protein that is tightly bound to either a small molecule (Johnston *et al.*, 1995) or another protein (Lee *et al.*, 2001) that prevents its unfolding will prevent entry into the proteasome, and impair degradation. The action of the ATPases on the 19s cap presumably aids to unravel the protein and thread it into the catalytic channel. The second requirement at the proteasome is the removal of the multi-Ub chain from the substrate. The proteasome contains resident Ub C-terminal hydrolases (UCHs) that will cleave the bond between the epsilon amino group of the acceptor lysine on the substrate and the C-terminal glycine residue of the proximal Ub (Leggett *et al.*, 2002). This activity releases the multi-Ub chain,

and the chain is then immediately broken down into free Ub again by the action of ubiquitin proteases (UBPs) and isopeptidase T in the cytoplasm (Amerik *et al.*, 1997). In summary, the affixing of a Ub tag to a substrate by a highly specific recognition cohort of enzymes serves to deliver substrate to a massive degradation machine that will bind the tag, unfold the substrate, and thread it into its proteolytic mouth, while recycling the enzymes involved in delivery.

As mentioned above, the proteasome mouth is like the iris of a camera—in that the proteasome central cavity does not remain open to the cytoplasm, but rather must be opened by both (1) the binding of the ubiquitinated substrate to specific subunits on the proteasome, and (2) conformational changes in the subunits of the proteasome that allow access of substrate to the central cavity/cylinder. The selection of protein as a potential substrate occurs through the highly conserved eukaryotic adaptation of the addition of Ub moieties. In addition to the destruction of cytoplasmic protein, the proteasome pathway is also responsible for the selective degradation of proteins in the secretory pathway via a process that has been termed ER associated degradation (ERAD) (Kostova and Wolf, 2003) (**Figure 9-1; see color insert**). In contrast to the prevailing view that transit through the secretory pathway is purely vectorial towards the plasma membrane, the concept of ERAD suggests that a protein that enters the ER still be subject to quality control mechanisms. If defective, the protein can be sent back (retrotranslocated) to the cytoplasm for destruction by proteasomes. One of the first proteins to be characterized as an ERAD substrate was the cystic fibrosis transmembrane conductance regulator (CFTR) protein (Ward *et al.*, 1995). Both the wild-type and the $\Delta 508$ mutant associated with cystic fibrosis disease are degraded by the Ub–proteasome pathway (Ward *et al.*, 1995). In the case of the mutant $\Delta 508$ CFTR, almost 100% of the mutant protein is targeted to the proteasome, while 50% of wild-type protein is degraded by the Ub–proteasome. The fact that the wild-type enzyme is also turned over suggests either the cell surface expression of the protein is highly regulated, or the protein is very difficult to fold correctly. Early experiments to understand the electrophysiological characteristics of CFTR involved the generation of stable cell lines expressing high levels of the CFTR protein. In the case of the wtCFTR or $\Delta 508$ mutant, it was not possible to obtain cells with greatly enhanced cell surface expression of the protein, and intracellular localization experiments revealed the protein had accumulated in the cells (Ward, Harrington, and Kopito, unpublished data). In parallel, pulse–chase experiments revealed that both of the CFTR proteins had a short half-life, and that the half-life could be greatly prolonged by the addition of proteasome inhibitors (Ward *et al.*, 1995). Proteasome inhibition also lead to the accumulation of CFTR protein in the insoluble fraction of cellular extract (Ward *et al.*, 1995; Johnston *et al.*, 1998). Because CFTR is a 12-transmembrane domain-spanning protein, it was of interest to understand if the insoluble CFTR protein accumulated in the context of the ER, or if it accumulated in the cytoplasm en route to the mouth of the proteasome. The use of GFP–CFTR expressing stable cell lines facilitated the use of fluorescent techniques to follow the fate of the CFTR molecules after inhibition of the proteasome with lactacystin or inhibition of lysosomes with ammonium chloride or E-64. In every GFP–CFTR expressing cell, it was possible to see a single large accumulation of fluorescent protein only after lactacystin treatment. This GFP protein was reactive to CFTR antibodies as well as Ub. The GFP signal was examined in the context of other intracellular markers for the Golgi, ER, and lysosomes, but did not colocalize with any of these intracellular markers. Electron microscopy of GFP–CFTR expressing cells revealed electron-dense accumulations of CFTR immunoreactive protein in the region of the centrosome, a notably cytoplasmic localization. As a result of these observations we decided to term the intracellular structure an “Aggresome” to reflect the novel localization in the cytoplasm, as well as the fact that there is a distinct process for its formation (Johnston *et al.*, 1998) (see later) The dislocation of membrane proteins into the cytoplasm of cells is likely to lead to the association of hydrophobic domains and rapidly lead to protein aggregation in the absence of timely degradation by the proteasome. Importantly, it was also established that simple overexpression of a

proteasome substrate can be sufficient to overwhelm the capacity of the proteasome and lead to aggresome formation. This concept suggests that proteasome substrate competition can delay the degradation of a protein sufficiently to allow formation of aggregated species. The crucial concept of substrate competition is discussed more fully in the Section III of this chapter.

It is also possible that cytoplasmic proteasome substrates, when targeted for degradation, become partially unfolded and vulnerable to aggregation if the degradation process is interrupted. As evidenced by the studies of the superoxide dismutase (SOD1) protein (Johnston *et al.*, 2000) among others (Garcia-Mata *et al.*, 1999), it is clear that not only membrane proteins that are proteasome substrates can form aggresomes, but a variety of cytoplasmic proteins as well. Protein folding studies suggest that point mutations in proteins can destabilize the final folded form of a protein. Also, as described in **Figure 9-1; see color insert**, there are many ways that a protein can become a substrate of the proteasome pathway. Protein damage, posttranslational modifications, phosphorylation, oxidation, genetic mutations, incomplete translation products, regulatory proteins, or overexpressed proteins, to name a few, can create proteasome substrates. The inability of the proteasome pathway to degrade substrates in a timely fashion can lead to protein aggregation, either through the inappropriate exposure of hydrophobic domains, or due to the hydrophobic patch on a multi-Ub ladder. Eventually, these aggregates can grow to a size that creates an impediment to the normal sieving of the cellular cytoplasm. Cytoplasmic sieving is an idea that describes the cytoarchitecture of cells and the capacity for molecular mobility through microdomains in the cytoplasm (Luby-Phelps, 2000). My “aggregate sieving” hypothesis is suggested from the high-powered digital deconvolution data, as well as electron microscopic data (Johnston *et al.*, 1998) of aggresomes. These images demonstrate that the microaggregates that coalesce at the aggresome are very similar in size. If the only requirement for incorporation into an aggresome were misfolding, or a Ub ladder (see HDAC6 discussion in Section 1.2) the aggresome would be predicted to be composed of heterogeneous aggregates. It is my opinion that the aggresome represents a cellular response to the accumulation of excessive misfolded protein, and the delivery of this aggregated protein to the aggresome represents an attempt to alleviate the crowding of the cytoplasm of a stressed cell. This response can allow for normal cellular functions to continue, even during stress. An alternative hypothesis worth testing, is that the HSP70–CHIP E3 ligase is capable of directing misfolded, undegradable protein to the MT motors (see below) for relocation in the cytoplasm and that the HSP70–CHIP complex recognizes substrates based on size or aggregation status.

3.2. Aggresomes Can Be Ub Positive or Ub Negative

Protein aggregation occurs when nonnative protein structures associate and adopt conformations leading to lower energy states than misfolded monomers. As discussed briefly in the Introduction, the proteasome is responsible for the elimination of misfolded and damaged proteins in cells. The proteasome can operate in a Ub-dependent and -independent mode. The 26S proteasome contains specialized caps for the recognition of Ub substrates, while the 20S core proteasome particle can degrade unfolded substrates without ubiquitination (Wenzel and Baumeister, 1995). Therefore, the proteasome degrades both membrane and cytoplasmic proteins, and can be Ub dependent or independent. For example, GFP-250 (Garcia-Mata *et al.*, 1999), mutants of SOD1 (Johnston *et al.*, 2000), and ornithine decarboxylase (Murakami *et al.*, 1996) are Ub-independent proteasome substrates, and CFTR (Ward *et al.*, 1995) and connexin 50 (Musil *et al.*, 2000) are two Ub-dependent proteasome substrates. GFP-250, SOD1 mutants, and CFTR form aggresomes when overexpressed in cells. (The ability of ornithine decarboxylase to form Ub-negative aggresomes has not been assessed.) Predictably, the GFP-250 aggresomes are not Ub positive (Garcia-Mata *et al.*, 1999), while the CFTR aggresomes are (Johnston *et al.*, 1998). It is likely that the accumulation of Ub in aggresomes reflects

the requirement for ubiquitination for the protein that comprises the majority of the aggregated protein in an aggresome. The microaggregates that accumulate in the cytosol of GFP–CFTR expressing cells after MT disruption are Ub positive (Johnston *et al.*, 1998), suggesting that the addition of Ub occurs at the level of the individual protein as targeted to the mouth of the proteasome. GFP-250 also accumulates as microaggregates after MT disruption, and these aggregates are Ub negative. These two observations argue against a model whereby Ub–proteasome pathway components are recruited to the area of the aggresome to ubiquitinate and degrade protein. If the latter were the case, all aggresomes would be Ub positive, because the presence of the aggresome would induce the ubiquitination of protein as it accumulated in the aggresome. Furthermore, the evidence from the studies of the HDAC6 protein (see Section 3.3), and the demonstration of substrate at peripheral cellular sites prior to movement on MTs for concentration into aggresomes, supports the idea that microaggregates of protein are harvested from the periphery of cells by recognition of size and/or ubiquitination, and the resulting aggresomes reflect only whether the misfolded protein required ubiquitination for its degradation.

3.3. Aggresome Formation Is an MT and Dynein–Dynactin-Dependent Process

Because the formation of pericentrosomal GFP–CFTR aggresomes was apparent in every cell in a field of cells, it suggested that there was a distinct mechanism responsible for the concentration of protein in one cellular location. It is highly unlikely that the newly ER-dislocated GFP–CFTR protein, in the absence of a specific pathway, would result in a single aggregate in the same place in every cell. This observation suggests a directed mechanism to move protein aggregates. Because the majority of intracellular transport occurs through MTs and MT-dependent motor proteins, we examined the role of MTs in the formation of aggresomes. The addition of MT disrupting agents lead to the appearance of a constellation of microaggregates distributed randomly throughout the entire cytoplasm of all of the cells (Johnston *et al.*, 1998). The disruption of the actin network did not have any effect on the formation of a single cellular aggresome. The disruption of the accumulation of GFP–CFTR protein into a single aggresome did not change the solubility of the protein. In other words, the GFP–CFTR partitioned to the insoluble fraction of the cellular extract regardless of the presence of a functional MT network. These results suggested that there is a MT-dependent process that gathers small insoluble aggregates of protein and actively moves them along MTs. This idea was confirmed in a modified MT assembly assay in which microaggregates of GFP–CFTR protein cosediment with MTs. Electron microscopy confirmed that the CFTR was present as proteinaceous attachments along the wall of the MTs (Johnston *et al.*, 2002). To determine if the retrograde movement of insoluble protein occurred through the action of the retrograde MT motor protein complex dynein–dynactin, we overexpressed the p50 subunit of the dynactin complex. This protein has been shown to disrupt the dynein–dynactin transport of cargo on MTs (Burkhardt *et al.*, 1997), and is known as p50dynaminin. Consistent with this described function of p50dynaminin, overexpression of p50dynaminin resulted in a similar constellation of microaggregates as seen when the MT network was disrupted (Garcia-Mata *et al.*, 1999; Johnston *et al.*, 2002). We have observed an accumulation of dynein and dynactin in aggresomes, although they do not accumulate as insoluble protein (Johnston *et al.*, 2002). Recently, the histone deacetylase protein 6 (HDAC6) was shown to serve as a linker for ubiquitinated substrate and the dynein motor (Kawaguchi *et al.*, 2003). HDAC6 has been implicated in the dynein-dependent transport of cargo on MTs (Hubbert *et al.*, 2002), and its mechanism of action may be related to the still unresolved role of acetylated tubulin/MTs (Matsuyama *et al.*, 2002). The HDAC6 did not facilitate the transport of nonubiquitinated substrates (Kawaguchi *et al.*, 2003), and therefore does not serve as a general “aggregate-dynactin” linker (see Figure 9-3; see color insert).

However, it does provide an important link to further understanding of the aggresome formation pathway, and supports the notion that aggresome formation is an active mechanism. It may be that there is a bipartite signal for aggresome formation: one component is a “sensor” of size of an aggregate (e.g., a size that begins to impede the normal sieving of the cytoplasm). Another component could be a Ub ladder (Ub-dependent substrate) or other protein (e.g., a multimer of HSPs may signal the presence of an aggregate) in a non-Ub-dependent substrate. Recognition of these two signals (size and Ub/HSP) then function to recruit MT motors to remove the aggregate to a distal location. The HSP90/FKBP52 protein complex is involved in the movement of hormone receptors through the cytoplasm to the nucleus (Czar *et al.*, 1995). The FKBP52 (hsp56) has been shown to bind to HSP90 as well as to the MT motor protein dynein (Silverstein *et al.*, 1999) through the peptidylprolyl isomerase (PPI) domain (Galigniana *et al.*, 2001). It may be that a similar recognition motif is used for the translocation of hormone receptor complexes as is used for the clearance of microaggregates of nonubiquitinated protein. FK506, a compound that inhibits the PPI activity of FKBP52, does not inhibit the trafficking of steroid–hormone receptor complexes (Czar *et al.*, 1995), suggesting it is the binding of the dynein to FKBP52 and not PPI activity that is required for MT/dynein-dependent movement of the hormone receptors. Similarly, the compound 15-deoxyspergualin (DSG) binds to HSP90 and inhibits the nuclear translocation of NF κ B (Holcombe *et al.*, 2002), and it would be interesting to know if this compound also affected the retrograde movement of nonubiquitinated aggregates to aggresomes.

3.4. Aggresomes Formation at the Centrosome

The centrosome is composed of two centrioles and the pericentriolar matrix (reviewed in Andersen, 1999; Rieder *et al.*, 2001). The centrosome is the cellular site for the nucleation of MTs (Tassin and Bornens, 1999). Microtubules form one of the major scaffold matrices for cells, and the polymerization of $\alpha\beta$ -tubulin into MTs is facilitated by the centrosome (Zheng *et al.*, 1998). The biophysical formation of MTs creates an “endedness” or polarity to the MTs, in that the end of the polymer where net assembly of new subunits occurs is the “plus” end. The area where subunits are released is the “minus” end of the MT. Centrosomes lower the critical concentration for the polymerization of tubulin into polymers by “capping” the minus end of the MT. The reduction in the disassembly rate of tubulin at the minus end allows the net growth of the polymer to proceed rapidly. Microtubule motors function, in part, through the use of the directionality of the MT polymer. Kinesins are MT motor proteins that move cargo along MTs from the minus end of the MT to the plus end (Goldstein and Yang, 2000). Dyneins are MT motor proteins that move cargo from the plus end to the minus end (Vallee *et al.*, 2004). As described above, dynein–dynactin has been shown to regulate the formation of aggresomes in cells, by concentrating aggregated protein at the centrosome. Because the dynein motor moves protein to the minus end of the MT, and the centrosome is at the terminus of the minus end of the MT, it appears that aggregated protein accumulates at the centrosome because the dynein motor has deposited its cargo at the end of its route.

The centrosome of all cell types is not the same, and it is important to understand the differences in centrosomes between cell types to understand the formation (definition) of aggresomes in different cell types. For example, the organization and function of the centrosome in neurons is different than in epithelial, or rapidly dividing cells. In epithelial cells, the centrosome forms the minus-end stabilized, radiating array of MTs that are dynamic throughout the cell cycle (**Figure 9-2; see color insert**). In neurons, the centrosome also serves as a nucleating center for the formation of MTs; however, it does not serve as an anchor for the extended MT cytoskeleton that extends through the axon and dendrites. Rather, after MTs are nucleated and grown to a certain extent, they are then transported down the length of the axon/dendrite, where they are added to a growing end of a MT

(Ahmad and Baas, 1995; Baas, 2002). In this regard, the neuronal MT network is considered to be discontinuous in that a given MT cannot be traced back to a minus end embedded in the centrosome as it is in epithelial cells (**Figure 9-2; see color insert**). It is probably for this fundamental difference that aggresomes in neurons can either be in the cell body, or in neurites, whereas in epithelial cells, aggresomes are almost invariably at the centrosome. Although it has been suggested that Lewy Bodies are aggresomes by the nature of colocalization of γ -tubulin and α -synuclein, the cell body of a neuron is extremely limited, and almost any inclusion could overlap with the centrosome. A more informative study would be directed at understanding the process of Lewy body formation, and a demonstration of a requirement for dynein–dynactin in that process (mechanisms of aggresome formation is discussed more thoroughly in Section III).

Centrosomes have been suggested as a central cellular location for Ub-proteasome degradation pathways, and proteasomes have been shown to localize to centrosomes after aggresome formation (Wigley *et al.*, 1999). Moreover, proteasomes have been coisolated during centrosomal preparations (Fabunmi *et al.*, 2000). However, localization studies have demonstrated that active proteasomes exist relatively homogeneously throughout cells, including the cytoplasm and the nucleus (Brooks *et al.*, 2000). Furthermore, studies in neurons have demonstrated that synaptosomes (Ehlers, 2003), as well as isolated growth cones (Campbell and Holt, 2001), contain all of the machinery for Ub–proteasome pathway degradation. Because the synaptosomes and isolated growth cones are no longer attached to the cell body, these studies demonstrate that independent of a cell body, Ub–proteasome function can proceed normally. In fact, isolated growth cones can still respond appropriately to chemical cues for attraction and repulsion, and these functional responses require Ub–proteasome degradation (Campbell and Holt, 2001).

One observation about aggresomes and centrosomes that has not yet been studied is the consistent characteristic deformation of the nuclear envelope after aggresome formation. Nucleus–centrosome complexes can be isolated from cells, and high-resolution electron micrographs of these preparations reveal a proteinaceous linkage of centrosome/pericentriolar matrix to the nuclear periphery (Maro, 1980). This association requires actin and MT, as cytochalasin B and nocodazole, respectively, dissociate nucleus and centrosome (Mitchison and Kirschner, 1986). Recently, genetic studies identified ZYG-12 as a centrosome–nucleus-linking protein (Malone *et al.*, 2003). It is interesting to note that the efficient isolation of aggresomes was achieved with a modification of a nuclei isolation procedure (Johnston *et al.*, 1998). Because of this stable association, it is surprising that aggresome formation deforms the nuclear envelope. This suggests that the aggresome formation has not disrupted the tight association between nucleus and centrosome, and further implies that the centrosome may have a more stable position in cells than the nucleus.

3.5. Aggresomes Are Associated with Rearrangements in IFs

Cellular organization and structure is maintained in part by the dynamic scaffold provided by three filament systems: actin, MTs, and IFs. The actin cytoskeleton is composed of polymers of f-actin microfilaments (7 nm) (Moon and Drubin, 1995). MT networks are composed of α , β -tubulin to form 25-nm fibers (Desai and Mitchison, 1997). Intermediate filaments are composed of cell-type-specific proteins to form filaments of intermediate diameter (8–12 nm); hence, the name IFs. IFs can be divided into at least six classes, with only the nuclear lamin class being expressed in all nucleated cell types (www.cytoskeleton.comaif.htm) (Skalli and Goldman, 1991). The remaining classes of IFs are expressed in a tissue/cell-type-specific fashion. For example, the IFs in glial cells are composed of glial fibrillary acidic protein (GFAP), neurons express neurofilament (NF) proteins, while the IF of smooth muscle cells are composed of desmin (as well as detectable amounts of vimentin and cytokeratin) and mesenchymal cells, and tissue culture cells contain vimentin (Skalli and Goldman,

1991). Finally, most epithelial cells contain 10-nm filaments of keratin. Although there are many classes of IF proteins, this review is focused on the types III, IV, and VI, as these are the types of IF proteins that have been demonstrated to become rearranged during aggresome formation. The function of IFs is still not altogether clear; however, it does appear that they play a role in the stability of cells during mechanical stress (NF, Leterrier *et al.*, 1996; and desmin, Wede *et al.*, 2002). One commonality of these 10-nm filament systems is that they are substrates for phosphorylation, and the level of phosphorylation can regulate the cellular distribution of the IF filaments (Goldman *et al.*, 1999; Chou *et al.*, 2003; Kawajiri *et al.*, 2003). In general, hyperphosphorylation of IFs leads to a condensation of the filaments into thick fibers. In the case of vimentin, which is a denizen of dividing cells, the phosphorylation of vimentin leads to its redistribution around the centrosome during early phases of mitosis (Chou *et al.*, 1991). Although it is now a well-established correlate of aggresome formation, the discovery of rearranged vimentin during aggresome formation was a bit serendipitous. Electron microscopy of CFTR aggresomes revealed that the electron-dense aggregates identified by immunogold-labeled CFTR antibodies were surrounded by a dense network of 10-nm filaments. This observation led to immunofluorescence experiments to determine the identity of the filaments. As described (Johnston *et al.*, 1998), vimentin immunofluorescence revealed a circumferential ring around aggresomes of CFTR. This rearrangement occurred whether the aggresomes were formed from overexpression of misfolded CFTR, or by pharmacological inhibition of the proteasome in CFTR expressing cells (Johnston *et al.*, 1998). Importantly, it was also shown that the vimentin reorganization occurred in naïve cells (i.e., not overexpressing any misfolded proteasome substrate), suggesting that proteasome activity, and not a specific association of vimentin with CFTR, resulted in the observed changes in vimentin dynamics. Because the proteasome is intimately linked to cell-cycle progression, and vimentin dynamics change throughout the cell cycle, it may be that regulatory factors that determine IF dynamics are regulated by the proteasome pathway. Some factors of this type have recently been demonstrated for vimentin, GFAP (Matsuoka *et al.*, 1992; Sekimata *et al.*, 1996), desmin (Kawajiri *et al.*, 2003), and NF (Sun *et al.*, 1996; Grant *et al.*, 2001). It has also been suggested that IFs can be anchored at the centrosome (Mitchison and Kirschner, 1986), providing an intimate connection to the cell cycle as well as an intriguing role in the positioning of centrosomes in cells.

As described in Section II, the presence of reorganized IFs is a characteristic of many pathological conditions. These same conditions are also characterized by the presence of large intracellular inclusions, suggesting that mechanisms of aggresome formation may play a role in the development of these disease states. Alternatively, the eventual development of a disease state may reflect the inability of a cell type to accommodate a persistent constraint on proteasome activity. In this regard, it must be emphasized that the previous statement does not suggest that disease arises from a global change in proteasome activity. On the contrary, the development of specific disease states that manifest with specific symptoms, but have a common pathological morphology argues that it is only in specific cell types that a decrease in proteasome capacity occurs (or a persistent constraint on activity). This concept is discussed more fully in Section III, Mechanisms of Aggresome Formation. However, it is likely that the consistent association of a rearranged IF network with aggresomes reflects a constraint on a common parameter downstream of proteasome activity.

4. Examples of Aggresomes in Human Health and Disease

The Ub-proteasome pathway plays a role in the regulation of normal protein half-life as well as the targeted elimination of damaged proteins (see above and **Figure 9-1**; see color insert). The generation of aggresomes in cells can result from an inhibition of proteasome catalytic activity, as

well as an overabundance of proteasome substrate (discussed in detail in Section III, Mechanisms of Aggresome Formation). This section discusses recent examples of aggresomes in both health and disease, and is summarized in **Figure 9-3; see color insert**.

First is a discussion of aggresomes in normal developmental processes. Interestingly, new studies indicate that the degradation of particular proteasome substrates can be delayed by the detour of substrates to a transient aggresome. These transient aggresomes are not linked to any particular cellular pathology; rather, they play an important role in normal development. Dendritic cells (DCs) form a dendritic cell aggresome-like structure (DALIS) as they mature into professional antigen presenting cells (Lelouard *et al.*, 2002). Glutamate receptors (GluR1) (Serrando *et al.*, 2002) and serotonin (5-HT₇) receptors (Muneoka and Takigawa, 2003) each form aggresomes in neurons during specific stages in development.

In the second part of this section, a multitude of data is discussed linking the pathological hallmarks of neurodegenerative disease, insoluble protein inclusions that are generally Ub positive, to aggresomes. Much of this data derives from mechanistic studies of aggresome formation after cellular expression of proteins that have been genetically linked to specific pathological disorders. For example, SOD1 in amyotrophic lateral sclerosis (ALS) (Johnston *et al.*, 2000), prion protein in Crutzfeldt-Jacob disease (CJD) (Ma and Lindquist, 2001), Huntingtin in Huntington's disease (HD) (Waelter *et al.*, 2001), PMP22 in Marie-Charcot-Tooth (Ryan *et al.*, 2002), presenilin in Alzheimer's disease (AD) (Johnston *et al.*, 1998; Ingano *et al.*, 2000), and α -synuclein/parkin in Parkinson's Disease (PD) (Dauer and Przedborski, 2003). In addition, myopathies (Ito *et al.*, 2002), hepatic disease (French *et al.*, 2001), cataracts (VanSlyke *et al.*, 2000), episodic ataxia-1 (Manganas *et al.*, 2001), lung disease (Kabore *et al.*, 2001), myoclonus epilepsy of the Lafora type (Ganesh *et al.*, 2002), and Wilson's Disease (Harada *et al.*, 2001) also appear to have specific cases that have arisen from defects in protein degradation and abnormal trafficking. In almost all of these studies, the common denominator for aggresome formation is a dysregulation of protein folding and/or proteasome degradation. The exception to this rule is the replication of virus in viral factories, and this remarkable exploitation of a normal cellular pathway allows efficient virus proliferation. Undoubtedly, the list will grow longer as more human genetic data emerges from population-based studies of disease. Many missense mutations that do not introduce a stop codon can disrupt the normal folding of a protein, and the majority of the disease examples below are a result of missense mutations in a particular gene. Obviously, not all disease can be reduced to a simplistic summary, and it is suggested that interested readers make use of the review articles indicated for a more thorough analysis for each of the complex disorders discussed below.

4.1. Aggresomes That Form as Part of a Normal Process

4.1.1. DALIS (Dendritic Cell Aggresome-Like Induced Structures)

Dendritic cells are involved in regulation of the immune response and undergo a process of maturation in response to inflammatory stimuli. At maturation, they are involved in the initiation of antigen-specific immune responses (Banchereau and Steinman, 1998). The proteasome plays a central role in the delivery of peptides to MHC class I molecules and subsequent antigen presentation on the cell surface (Rock *et al.*, 1994; Rock and Goldberg, 1999). During the process of maturation, dendritic cells form transient Ub-positive inclusions that have been termed DALIS (Lelouard *et al.*, 2002). These inclusions are similar to aggresomes in that they are Ub positive and can be induced (in this case after lipopolysaccharide treatment). They differ from "classical" aggresomes in a few important ways. For example, DALIS do not form at the centrosome, do not require MTs for formation, and are not associated with a rearrangement of vimentin, although there is an increase in ubiquitinated protein (Lelouard *et al.*, 2002). Interestingly, this difference may lie in the nature of the DC and its role in

antigen presentation. It has been shown that DCs during maturation do not have a temporary inhibition of the proteasome (Lelouard *et al.*, 2002), but rather a specific targeting of newly synthesized defective ribosomal products (DRiPs) to a DALIS until maturation of the DC is complete (Lelouard *et al.*, 2004). After DC maturation, the DALIS disappears. The addition of proteasome inhibitors to a DC after a DALIS has formed prevents the disappearance of the DALIS—revealing the requirement for a functional proteasome system to process the DRiPs for antigen presentation (Lelouard *et al.*, 2004). Although it may seem that the DALIS and the aggresome are completely different intracellular mechanisms, it may be that the DALIS has revealed a cellular mechanism to segregate DRiPs under conditions of intracellular stress in nonimmune cells. For example, the unfolded protein response (UPR) has been shown to regulate translation initiation factors to decrease translation in times of accumulation of misfolded proteins in cells (Patil and Walter, 2001). There may also be a mechanism similar to the formation of DALIS during UPR that prevents the release of new proteins into an already stressed system, by targeting newly synthesized proteins to a transient “holding structure.” Cotranslational protein degradation has been demonstrated (Turner and Varshavsky, 2000); however, the intracellular redistribution of ubiquitinated proteins occurring during this response was not examined. Dystrophic neurons may also undergo similar DALIS-like mechanisms. For example, it may be beneficial to suspend delivery of new protein to a site distal to the cell soma if the distal location is not functioning normally. In fact, the accumulation of cell body inclusions versus neuritic inclusions in diseased neurons may reflect such a process (see Mechanisms of Aggresome Formation). Through the use of techniques described to form DALIS (Lelouard *et al.*, 2004) it should be possible to study this process in neurons and other cell types—specifically in regard to the experimental generation of DRiPs and a study of their fate.

4.1.2. Glutamate Receptor Subunit 1 (GluR1) Aggresomes

Glutamate and glutamate receptors (GluR) in neuronal tissue comprise one of the cornerstones of neuronal function and have been studied extensively (Dingledine *et al.*, 1999). Glutamate receptors of the AMPA type are composed of subunits, and the composition of AMPA subunits can be correlated to certain cell types (Borges and Dingledine, 1998). The appropriate assembly of the AMPA subunits is required for the cell surface expression of the AMPA receptors (Malinow and Malenka, 2002). During the course of an immunocytochemical study of AMPA GluR subunits in developing rat spinal cord, it was noticed that the GluR1 subunit was found in discrete cytoplasmic foci in the cell body of GABAergic interneurons between embryonic day 19 and postnatal day 17 (Serrando *et al.*, 2002). These GluR1 foci are found next to the nucleus and stain positive for Ub, HSP70, and 20s proteasome. Immunofluorescence techniques revealed that the neuronal IFs α -internexin and NF-H were shown to coincide with the GluR1 inclusions (Serrando *et al.*, 2002). However, the electron microscopic data did not demonstrate a clear circumferential or bundled collapse of IF in the vicinity of the inclusions. The inclusions were transient, and did not correlate to cell death as a part of programmed cell death during development (Serrando *et al.*, 2002). Similar to DALIS, also a temporary structure, it may be that nonpathological aggresomes form as a part of developmental processes, and these transient aggresomes are not accompanied by IF collapse/reorganization. In this regard, it would be interesting to know if DRiPs, and/or cotranslational degradation of specific transcripts of GluR1 receptors, are not only regulated temporally through development, but also spatially within specific cell types. It may be that the temporary sequestration of the GluR1 subunits during development serves to restrict the delivery of the functional AMPA receptors to the cell surface, and provides a temporary mechanism to regulate the ability of developing neurons to respond to glutamate. Further studies of developmental systems will be important to determine if transient aggresome

formation is a distinct developmental mechanism and if it plays any role in the ability of developmental systems to rapidly respond to stimuli during development.

4.1.3. Stigmoid Bodies: 5-HT₇ Receptors and Aromatase

The “Stigmoid Body” (Shinoda, 1994) occurs as a transient intraneuronal inclusion, and is observed during days 1–21 of both male and female rats during brain development. Stigmoid bodies stain positive for aromatase (Shinoda, 1994), as well as the 5-HT₇ receptor (Muneoka and Takigawa, 2003). The acquisition of sexual dimorphism and sleep–wake regulation occurs in the brain regions demonstrated to show Stigmoid bodies. 5-HT₇ receptors and aromatase are intimately involved in these processes, suggesting that the Stigmoid body provides a regulatory mechanism to control the levels of crucial proteins involved early in the development of the brain at specific times during development. It would be interesting to determine if the Stigmoid bodies are Ub positive, as this would suggest a common mechanism for DALIS, GluR1 aggresomes, and Stigmoid bodies formation as transient aggresomes. Interestingly, Stigmoid bodies stain positive for HAP1 (Gutekunst *et al.*, 1998). HAP1 is known to bind to p150glued, and has been suggested to be part of the dynein–dynactin motor (Li *et al.*, 1998). If DALIS and GluR1 aggresomes also stain positive for HAP1, it may be that the HAP1 provides at least part of a substrate recognition pathway to direct protein to transient aggresomes. HAP1, although originally found to bind expanded CAG of HTT, does not accumulate in the HTT-positive inclusions (Gutekunst *et al.*, 1999), and this may suggest that one role of HAP1 is restricted to the delivery of cargo to transient aggresomes and can distinguish particular motifs on appropriate substrates.

4.2. Aggresomes That Form as Part of a Disease Process

4.2.1. Aggresomes and Viral Factories

Viruses are infective agents that are composed of protein and nucleic acid. The entry of viruses into cells and the successful replication of virus rely upon exploitation of normal cellular processes. Large DNA viruses use the spaciousness of the cellular cytoplasm for their assembly into mature particles via a process/structure known as viral factories. Studies of the African swine fever virus (ASFV), a large DNA virus, revealed that the successful replication of this virus relies upon mechanisms similar to the formation of aggresomes (Heath *et al.*, 2001). Specifically, after entry into the cells and uncoating (Valdeira *et al.*, 1998), the nucleoprotein core particle becomes cytoplasmic (Valdeira *et al.*, 1998). The nucleoprotein particle was shown to move retrograde along MTs, to the centrosome where the particles concentrated, and this movement required dynein and dynactin, as the overexpression of p50 dynamitin prevented particle accumulation at the centrosome (Heath *et al.*, 2001). Nocodazole disruption of the MT network also prevented nucleoprotein particle transit to the centrosome (Heath *et al.*, 2001). Importantly, the authors demonstrated the formation of the aggresome was crucial for the replication and transition to late gene expression of the virus (Heath *et al.*, 2001). In the absence of nucleoprotein particle accumulation at the centrosome (either nocodazole treatment or overexpression of p50dynamitin), only the abundant early gene p30 of the nucleoprotein particle could be detected. However, in the presence of a functional MT network and particle accumulation at the centrosome, p73, an indication of transition to late gene expression was clearly present in the cells (Heath *et al.*, 2001). The centrosomal viral factories were also shown to be surrounded by a cage of vimentin filaments (Heath *et al.*, 2001), another strong similarity to aggresomes. Frog virus 3 (FV3), is a virus that is structurally related to ASFV and has been shown to form a cage if collapsed

vimentin around FV3 assembly sites (Chen *et al.*, 1986). The collapsed vimentin after FV3 infection was shown to contain a fourfold increase in phosphorylation (Chen *et al.*, 1986). It could be that the virus infection disrupts the cell cycle of the host cell and the collapse of vimentin is a consequence of the global cellular disruption. The Epstein-Barr virus has been shown to contain proteins (Epstein-Barr nuclear antigen-1, EBNA-1) with repeat sequences that can inhibit proteasome activity (Levitskaya *et al.*, 1997) and inhibition of the proteasome will impair progression through the cell cycle. As discussed above, it is likely that cell cycle mechanisms regulate the rearrangements of IFs that are concurrent with aggresome formation. Therefore, it would be interesting to know if the ASFV also contains a protein that will allow infected cells to escape immune system detection by inhibition of proteasome activity and MHC Class I antigen presentation (as is the case for Epstein-Barr virus) (Levitskaya *et al.*, 1997). It is also important to note that a second mechanism for immune system evasion for Epstein-Barr virus infected cells is that EBNA-1 repeats inhibit translation of its own mRNA, resulting in the accumulation of EBNA-1 DRiPs (Yin *et al.*, 2003). DRiPs were discussed above (see DALIS), and may represent yet another mechanism for targeting undegraded proteasome substrates to a cellular microdomain.

The localization of viral nucleoprotein particles concentrated at the centrosome, a requirement for MTs, dynein, and dynactin, as well as a collapse of vimentin filaments all indicate that the formation of viral factories is similar to aggresome formation. Whether an inhibition of proteasome activity is a fundamental mechanism that underlies these similarities and leads to cell cycle arrest is not yet established. It is clear that a cellular response to viral infection leads to a similar reorganization of intracellular components as observed after the accumulation of misfolded proteins. Moreover, the potential involvement of DALIS/DRiPs in regulation of protein access to the proteasomes provides us with a continuum of regulation of substrate access to the proteasome. The proteasome pathway may have a way to identify and triage substrates, ranking them according to priority access to degradation. An alternative view would argue that specific cellular functions override the normal process of proteasomal processing and delay progression through the cell cycle. Finally, there could be changes that occur in cells as they go from pluripotent to terminally differentiated that requires a change in the cell cycle. For example, as the dendritic cell goes from a dividing immature DC to the mature DC, it ceases to divide. Likewise, virally infected cells cease dividing and neuronal cells are also postmitotic. The formation of aggresomes in cells has also been shown to arrest aggresome containing cells at the G2–M transition (Bence *et al.*, 2001). These processes are linked by the common observation of aggresomes, as well as cell cycle arrest. It will be interesting to know if the mechanisms that create proteasome impairment are similar or distinct, and if the mechanisms offer clues to pathological formation of aggresomes.

4.2.2. Mallory Bodies: Hepatic Disorders and Cytokeratin

Mallory bodies are Ub-positive inclusions found in liver cells in pathological liver disease. Mallory bodies can be induced to form in mice after a variety of insults, and recently the formation of Mallory bodies as an aggresome was reported (French *et al.*, 2001; Bardag-Gorce *et al.*, 2003; Riley *et al.*, 2003). Mallory bodies are pericentrosomal, Ub positive, and require MTs for formation. A protocol for the induction and isolation of mouse Mallory bodies has been reported (Zatloukal *et al.*, 2002). Compositional analysis using Maldi-TOF mass spectrometry of the isolated Mallory bodies has identified polyubiquitinated, hyperphosphorylated cytokeratin 8, Lamin B, p62 (Stumptner *et al.*, 1999), cytokeratin 18, cytokeratin 8, HSP70, and HSP 25 as major protein components (Zatloukal *et al.*, 2002). p62 is an Ub-binding protein (Vadlamudi *et al.*, 1996), and has been

shown to be induced during apoptosis as well as proteasome inhibition (Kuusisto *et al.*, 2001b). The role of p62 in Mallory bodies is unknown, although it is probably related to its Ub binding activity, as the overexpression of p62 alone did not result in inclusion body formation. It is notable that p62 can be found in a number of other aggresome diseases such as Lewy bodies in PD, Rosenthal fibers in astrocytoma, and neurofibrillary tangles in AD (Kuusisto *et al.*, 2001a, 2003; Zatloukal *et al.*, 2002). One hypothesis from this author posits that p62 is induced under conditions of proteasome stress and the p62 helps to prevent the hydrophobic patches on accumulating multi-Ub ladders from aggregating. This hypothesis could be tested directly via the use of siRNA for p62 to determine if aggresome formation was increased in the absence of p62, or alternatively, if p62 can slow the formation of aggregates. It has been proposed that Ub sequesters Ub binding proteins in aggregates, and that their depletion from the functional pool contributes to pathological processes (Donaldson *et al.*, 2003). Ubiquitin binding proteins are known to be involved in endocytic processes (Hicke and Dunn, 2003).

In light of the data suggesting that the chaperone $\alpha\beta$ -crystallin is involved in the turnover of Desmin (see myopathies), it would be interesting to know if $\alpha\beta$ -crystallin associates with Mallory bodies, or if HSP70 is acting as part of the CHIP-Ub ligase complex (Murata *et al.*, 2001) and is directly involved in targeting cytokeratin for degradation.

The protocol used for the isolation of Mallory bodies was stringent enough that it is likely the only proteins identified were insoluble proteins; however, it would be interesting to know the identity of soluble proteins that coisolated with the Mallory body. For example, even though dynein, p150 glued, and p50dynamitin are not insoluble in an aggresome, they do accumulate there (Johnston *et al.*, 2002), and it would be interesting to know if the same MT motors are operating in liver cells. This could be tested in the *in vitro* hepatocyte model (French *et al.*, 2001; Riley *et al.*, 2003) by the overexpression of p50 to disrupt dynein-dynactin function, which has been shown to disrupt aggresome formation (Garcia-Mata *et al.*, 1999; Johnston *et al.*, 2002). The frameshift mutant of Ub Ub+1 has been shown to be a constituent of human Mallory Bodies (McPhaul *et al.*, 2002), and can induce Mallory body formation *in vitro* (Bardag-Gorce *et al.*, 2003). Ub+1 has been shown to inhibit proteasome function (Lam *et al.*, 2000; Lindsten *et al.*, 2002), and is discussed in detail in Section III, Mechanisms of Aggresome Formation. This data suggests that proteasome inhibition *in vivo* can lead to Mallory body formation.

Transgenic mice that do not express cytokeratin 8 and also a strain that does not express cytokeratin 18 have been studied in relationship to induced Mallory body formation (Zatloukal *et al.*, 2000). The cytokeratin 8 knockout (KO) mice revealed a marked increase in sensitivity to liver insult, and did not form Mallory bodies (Zatloukal *et al.*, 2000). In contrast, the cytokeratin 18 KO mice were not increased in sensitivity to insult, and were able to form Mallory bodies (Magin *et al.*, 1998). The cytokeratin 18 KO mice also developed spontaneous aggresomes in liver cells as the animals aged (Zatloukal *et al.*, 2002). This study reveals two important concepts about the role of aggresomes: (1) the formation of an inclusion can be protective for cells; (2) that an imbalance in cellular constituents can lead to an age-dependent formation of an inclusion.

Because cytokeratin is an IF and is a major component of Mallory bodies, it demonstrates that rearrangements of cytokeratin are related to the Mallory body aggresome. This particular case suggests that derangement in IFs can cause aggresomes to form. Specifically, it appears that the ratio of cytokeratin 8 and 18 is critical for the appropriate generation of an IF network in liver cells, and a disruption of this ratio can lead to inclusion formation (Magin *et al.*, 1998; Zatloukal *et al.*, 2000). Because the liver is a major filter for toxins in the body, it is not surprising that Mallory bodies are formed from a variety of toxic insults in hepatocytes and potentially serve as a protective mechanism.

4.2.3. Retinitis Pigmentosa: Rhodopsin

Retinitis Pigmentosa (RP) defines a heterogeneous group of visual disorders that eventually lead to blindness through the degeneration of rod and photoreceptor cells in the retina of the eye (reviewed in Milam *et al.*, 1998). Almost 50% of autosomal dominant RP has been linked to mutations in the rhodopsin gene (Dryja *et al.*, 1990, 1991; Sung *et al.*, 1991a), and of these mutations, potentially half are mutations that prevent normal folding of the protein (Sung *et al.*, 1991b, 1993; Illing *et al.*, 2002). Rhodopsin is a seven-pass transmembrane photopigment protein, a G-protein-coupled receptor, that is present in high concentrations in the outer segment of photoreceptor cells (Okada *et al.*, 2001). Rhodopsin mutants P23H and K296E have been shown to be substrates of the Ub–proteasome pathway (Illing *et al.*, 2002; Saliba *et al.*, 2002), and overexpression of these mutants in cultured cells leads to the formation of pericentriolar, MT-dependent, IF-associated, Ub-positive aggresomes (Rajan *et al.*, 2001; Illing *et al.*, 2002; Saliba *et al.*, 2002). Curiously, the degeneration of the rod and photoreceptor cells has not been correlated to the presence of Ub-positive inclusions (Flannery *et al.*, 1989; Milam *et al.*, 1998). As discussed above for ALS/SOD protein and the Mallory Body, prior to the accumulation of microaggregates to create a visible aggresome, it is possible to detect SDS-stable (biochemically detectable) aggregates called insoluble protein complexes (IPCs). These IPCs precede overt aggresome formation, and likely represent the early accumulations of misfolded protein that may, in fact, be more detrimental to cellular function than coalesced microaggregates in aggresomes. In this regard, the absence of large inclusion bodies in RP may reflect the distinct architecture of the photoreceptor cell. The photoreceptor cell is involved in a variety of complex functions, and its intracellular organization reflects this multitude of functions. Please see Wolfrum and Schmitt (2000) for a thorough discussion of the transport mechanisms and architecture at work in the photoreceptor cell. The organization of the MT network in a photoreceptor cell holds particular interest to understand the lack of aggresomes in human RP. Specifically, photoreceptor cells are a complex polarized cell with an inner segment and an outer segment. The inner segment contains a synapse for the transmission of light information to rod cells, as well as the nucleus, Golgi, and ER and mitochondria. The outer segment contains the hundreds of rhodopsin-containing membrane disks that are responsible for responding to light. The inner and outer segments are connected by a thin connecting cilium [a nonmotile cilium, that is composed of MTs and Basal bodies (BB) structures similar to centrosomes]. The MTs emanate from the BBs, and the plus ends of the MTs extend from the BB to the synapse in the inner segment, and from the connecting cilium through the hundreds of rhodopsin-containing membrane disks in the outer segment. As discussed in Wolfrum and Schmitt (2000), the biosynthetic machinery in the photoreceptor cell is organized such that MT-based transport of newly synthesized rhodopsin moves from the plus end of the MT to the minus end to move from the biosynthetic machinery towards the outer segment—its ultimate destination. Most other cell types move material from biosynthesis to the cell surface using plus end-directed MT motors like kinesins (reviewed in Muresan, 2000). The movement of rhodopsin through the inner segment has been shown to require a direct link of dynein to the carboxyl-tail of rhodopsin through binding to Tectex-1 (Tai *et al.*, 1999). However, after rhodopsin has moved retrograde to the connecting cilium, there must be a change in the MT motor that moves it into the outer segment because the directionality of the MT changes from retrograde to anterograde. Myosin VIIa (Wolfrum and Schmitt, 2000) and KIF3A (Muresan *et al.*, 1998) are candidates for this movement. KIF3A, a kinesin that has been shown to be present in small amounts in the outer segment (Muresan *et al.*, 1998) may be involved in retrograde movement of misfolded aggregates out of the outer segment. However, there is no evidence for this as yet, and photoreceptor cells are not a facile *in vitro* system to study mutant rhodopsin aggresome formation (although elegant transgenic systems have been developed in *Xenopus*) (Tam *et al.*, 2000). It may be that the aggregation of mutant rhodopsin molecules occurs

at the connecting cilium/BBs and prevents the delivery of rhodopsin into outer segments. In fact, although it has been shown that RP is associated with loss of photoreceptor cells, it would be interesting to know if a time-course study of photoreceptor cells expressing mutant rhodopsin revealed a point prior to cell degeneration in which ubiquitinated rhodopsin aggregates were detectable. Current data for autosomal dominant mutants of rhodopsin in RP suggest that photoreceptor cells may lack the capacity to form aggresomes, and as a consequence, cannot readily protect themselves from the accumulation of misfolded rhodopsin aggregates—leading to profound cell loss. Like many of the other proteins discussed in this section, it appears that aggresome formation may play a protective role in delay of cellular degeneration.

4.2.4. Desmin-Related Myopathy (DRM): α , β -Crystallin

Desmin-related myopathy is an autosomal dominant disease characterized by large desmin and α , β -crystallin aggregates in skeletal and cardiac muscle (Goldfarb *et al.*, 2004). Mutations in desmin (Goldfarb *et al.*, 1998) and α , β -crystallin (Vicart *et al.*, 1998) are linked to the development of DRM. Desmin is an IF protein. α , β -Crystallin is a small HSP proposed to have chaperone functions (Klemenzt *et al.*, 1991; Kato *et al.*, 1999). It is expressed at high levels constitutively in lens and muscle tissue (Srinivasan *et al.*, 1992), and can be induced after stress in a wide range of cell types. The DRM related α , β -crystallin mutant R120G has been shown to form aggresomes in cells independent of desmin (Chavez Zobel *et al.*, 2003). The R120G α , β -crystallin has impaired *in vivo* function (Chavez Zobel *et al.*, 2003), which is probably due to misfolding of the protein because: (1) overexpression of chaperones HSPB8, HSP27 (Ito *et al.*, 2003), and wild type α , β -crystallin-reduced aggresome formation; (2) expression of the HSP70–ChiP1 ligase complex recognized and ubiquitinated the mutant R120G α , β -crystallin and decreased aggresome formation (Chavez Zobel *et al.*, 2003). Wild-type α , β -crystallin (and HSP27) was shown to localize to aggresomes formed in naïve HeLa cells treated with MG-132 and in NG-108 cells expressing the aggresome-forming protein GFP-250 (Garcia-Mata *et al.*, 1999; Ito *et al.*, 2002), and both HSP27 and wild-type α , β -crystallin were shown to accumulate in the insoluble material over time. Although this suggests that perhaps α , β -crystallin is a proteasome substrate, that if not degraded can form aggresomes, α , β and HSP27 did not accumulate as high molecular weight ubiquitinated species. The aggresomes formed by R120G α , β -crystallin are highly reactive for Ub (Chavez Zobel *et al.*, 2003), although a direct linkage of Ub to any form of α , β -crystallin has not been demonstrated. Aggresomes can be Ub positive or negative (see section I); however, this is the first example of an aggresome that is Ub positive formed from a protein that is not ubiquitinated. α , β -Crystallin is phosphorylated in a cell cycle-specific (Kato *et al.*, 1998) or stress-specific manner (Ito *et al.*, 1997); however, it is unclear what role this plays in the function of α , β -crystallin. Phosphorylation has been shown to be independent of aggresome formation (Ito *et al.*, 2002). α , β -Crystallin has recently been described to associate with FBX4 and confer Ub ligase activity (den Engelsman *et al.*, 2003). FBX4 is a component of the SCF Ub ligase complex that is involved in the recognition of phosphorylated substrate for Ub ligation by the SCF (Craig and Tyers, 1999). Interestingly, only the phosphorylated forms of α , β -crystallin interacted with FBX4 to “stimulate” the ligase activity. It is not clear if the association of α , β -crystallin and FBX4 is a cell cycle-specific event that results in activation of a crucial ligase activity, or if the mitosis-specific phosphorylation is a signal for the α , β -crystallin degradation. It would be interesting to understand the basic half-life characteristics for α , β -crystallin throughout the cell cycle, especially in regard to the phosphorylation status. Because the expression of FBX4 with R120G α , β -crystallin, or the phospho α , β -crystallin, resulted in a massive induction of ubiquitinated proteins (den Engelsman *et al.*, 2003), it would also be interesting to know if this coexpression resulted in the formation of aggresomes in cells using immunofluorescent techniques. As discussed in detail in Section III,

Mechanisms of Aggresome Formation, an increase in the abundance of misfolded protein destined for the proteasome (in this case, R120G or phospho $\alpha\beta$ crystallin) can create a substrate competition that eventually causes other proteasome substrates to aggregate and become insoluble. The fact that $\alpha\beta$ -crystallin is also a chaperone that is important for IF dynamics, as well as subject to its own regulated turnover, could confer a particular sensitivity to cells that rely on the function of $\alpha\beta$ -crystallin through the cell cycle.

4.2.5. Prion Disorders: Prion Protein

Prion disorders are a neurodegenerative condition that can be acquired or inherited (Prusiner, 1998). In both cases, the pathogenic agent is a misfolded form of the prion protein (PrP), a transmembrane protein in neurons whose function is not yet known (Prusiner *et al.*, 1998). Insoluble inclusions of a protease resistant form of the PrP (PrP^{sc}) accumulate in the brain and lead to cell death. Acquired prion disorders are thought to arise from exogenous misfolded PrP accumulation in brain tissue through ingestion of “infectious” prion material (Prusiner, 1998). Inherited forms of prion disease are known as Crutzfeldt-Jacob (CJD), Gerstmann-Straussler-Scheinker (GSS), and fatal familial insomnia (FFI). Over 25 different mutations in the prion gene (PRNP) have been linked to these familial prion diseases, and in general, these disorders are autosomal dominant, because many of the patients are heterozygous for the mutation (http://www.cyber-dyne.com/~tom/allele_review.html). Because the pathogenesis of prion disorders is clearly linked to the acquisition of a misfolded, protease resistant form of the protein, recent attention has been focused on understanding the biosynthesis of the mutant prion molecules and how the mutations lead to the deposition of insoluble prion protein in cells. The Y145stop mutation (Zanusso *et al.*, 1999) and the Q217R mutations (Jin *et al.*, 2000) associated with GSS are both substrates of the ERAD/proteasome pathway. The D177N PRNP mutation is one of the most common, and has been shown to rapidly form cytoplasmic inclusions, even in the absence of proteasome inhibitors (Ma and Lindquist, 2001). Only a small proportion of the D177N PrP protein is able to progress to any extent through the secretory pathway (Ma and Lindquist, 2001). V203I, E211Q and Q212P are also subject to ERAD and form aggresomes in cultured cell models (Mishra *et al.*, 2003). In all of these cases, the inclusions are formed from insoluble, ubiquitinated, deglycosylated PrP (Ma and Lindquist, 2001; Yedidia *et al.*, 2001). Interestingly, and consistent with other disorders discussed in this section, a proportion of the wild-type prion protein is also recognized for degradation by cytosolic proteasomes and can accumulate as insoluble, ubiquitinated material in cells (Ma and Lindquist, 2001; Yedidia *et al.*, 2001). In the absence of proteasome inhibitors wild-type PrP does not accumulate in cells unless a great excess of wild-type PrP is expressed in the cells, suggesting that the proteasome is extremely efficient in its degradation of ERAD substrates. The spontaneous aggregation of the D177N in cells to form aggresomes strongly suggests that this mutant rapidly adopts a conformation that is problematic for the unfolding activities of the proteasome (see Section III, Proteasome Competition) and leads to cytoplasmic insoluble inclusions. Because the PrP can adapt a particularly infectious conformation that can be accelerated by cellular conditions (Ma and Lindquist, 2002), timely degradation by the proteasome is essential for cellular health. The accumulation of PrP in the cytosol has been linked to toxicity and degeneration (Ma *et al.*, 2002). Cellular stress, mutation, or environmental insults that decrease the rate of proteasomal processing of substrates could allow the misfolded PrP molecules the opportunity to covert to the PrP^{sc} conformation. Likely, any process that disrupts the timely degradation of wild-type or mutant PrP forms would be expected to be detrimental. Alternatively, the presence of a rapidly aggregating mutant of PrP, like the D177N, could create a kinetic bottleneck at the proteasome, whereby the ATPase activity of the proteasome (Navon and Goldberg, 2001) unsuccessfully attempts multiple rounds of unfolding. PrP has been shown to form aggresomes in cells, and their formation

is MT dependent, associated with rearranged vimentin, and accompanied by the acquisition of protease resistant PrP (Ma and Lindquist, 2001; Cohen and Taraboulos, 2003). Interestingly, PrP aggresomes were shown to form after cyclosporin A treatment, and the resulting aggresomes were Ub negative (Cohen and Taraboulos, 2003). The PPI activity of FKBP is inhibited by cyclosporin A (Schreiber, 1991) and inhibition of PPI may lead to rapid PrP misfolding. As discussed above (Section I, 3.3, MTs) FKBP *may* mediate the movement of nonubiquitinated substrate in MTs to form aggresomes. Cyclosporin A does not affect the dynein FKBP interaction, and the PPIase activity is not required for dynein mediated transport (Schiene-Fischer and Yu, 2001), consistent with the accumulation of PrP aggresomes via an FKBP-mediated movement. The disruption of the PPI activity via cyclosporin can lead to misfolding of PrP and aggresome formation. It would be interesting to identify a drug that could disrupt the dynein–FKBP52 interaction but not inhibit the PPI activity, to separate the two distinct activities of FKBP52 protein.

4.2.6. Bunina Bodies: ALS and SOD1

Amyotrophic lateral sclerosis is characterized by a progressive degeneration of the upper and lower motor neuron systems (Valentine and Hart, 2003). Amyotrophic lateral sclerosis can be inherited or sporadic. In the case of sporadic disease, much less is known about the cause of the pathology; however, the degeneration is accompanied by the presence neuropathologically of Ub-positive inclusions in the spinal cord neurons known as Bunina bodies. The pathology is not restricted to spinal cord, and can be detected in various brain regions. So far, all identified affected brain regions contain Ub-positive inclusions, and these inclusions closely parallel associated clinical symptoms (Piao *et al.*, 2003).

In the case of inherited disease, Bunina bodies are also present, and may suggest a common molecular cascade as for the sporadic forms of disease. Mutations in the SOD1 gene have been identified and account for 15–20% of the inherited cases of ALS (Rosen *et al.*, 1993; Siddique and Hentati, 1995; Valentine and Hart, 2003). SOD1 is an enzyme important for the amelioration of oxidative byproducts produced by cellular chemistry. Antibodies to Ub and the SOD1 protein label Bunina bodies (Shibata *et al.*, 1996). One role for the dominant gain-of-function of SOD1 mutations could be a dominant negative ability of the mutant to inhibit wild-type SOD1 function. Mutants in SOD1 do not affect the activity of the wild-type enzyme, and the mutant SOD1 enzyme retains dismutase activity (Borchelt *et al.*, 1994, 1995). Moreover, animals that are completely absent the SOD1 enzyme do not develop ALS-like disease, suggesting loss of enzymatic function is probably not the basis of ALS (Bruijn and Cleveland, 1996). Another possibility is that the misfolded SOD1 induces a cellular stress response as the affected cells accumulate misfolded protein. Mutant forms of SOD1 are degraded by proteasomes (Hoffman *et al.*, 1996; Johnston *et al.*, 2000). The expression of SOD1 mutants in tissue culture cells results in the formation of “classical” aggresomes (Johnston *et al.*, 2000). These aggresomes are not highly Ub positive, and although high molecular weight forms of SOD1 mutant protein could be detected as SDS-stable aggregates, they did not correlate to Ub positivity (Johnston *et al.*, 2000). The availability of robust transgenic mouse models of mutant SOD1 ALS (Gurney, 1994; Cleveland *et al.*, 1996) has provided important insight into understanding disease progression. These transgenic mice express a genomic gene for SOD1 (containing disease-linked mutations in human SOD1 protein) and exhibit age-dependent and cell-type specific accumulations of insoluble aggregates of the SOD1 mutant protein (Gurney, 1994; Cleveland *et al.*, 1996). These animals also develop hindlimb weakness in a progressive fashion, eventually leading to immobility (and inability to feed themselves) and death. A temporal analysis of the lumbar portion of the spinal cords of these animals revealed that long before the development of Bunina bodies occurred, it was possible to detect high molecular weight aggregates of SOD1 protein (Johnston *et al.*, 2000). The

amount of these aggregates increased as the animals aged, and correlated to the development of pathological symptoms such as hindlimb weakness (Johnston *et al.*, 2000). These studies suggest that the Bunina body has formed after a long pathological process in which small microaggregates of protein have been creating a cellular strain for months or, in the case of humans, for years prior to the onset of clinically definable symptoms. An analysis of cDNA microarray experiments in late-stage human patients revealed that cellular stress pathways, including the proteasome pathway, are upregulated (Dangond *et al.*, 2004). Studies from transgenic mice (Olsen *et al.*, 2001) and cell culture (Kirby *et al.*, 2002) have produced results that implicate the same pathways. A recent proteomic study has provided similar results, and biochemically demonstrated that mutant SOD1 can negatively impact the proteasome pathway (Allen *et al.*, 2003). Interestingly, the human and transgenic mouse studies reveal a clear component of inflammation. Although this is not entirely unexpected in a degenerative disease, it is worth noting that any process that could impact the function of the proteasome can have an impact on the expression of cell surface proteins, as well as potential stress signals released from cells. The immune system operates, generally speaking, through the recognition of cell surface motifs and secreted molecules, and is highly sensitive to changes in these characters. A disease process that impacts the proteasome can produce subtle changes in cell surface and secreted proteins that could be recognized by normal immune surveillance in the brain long before the development of large intracellular inclusions. Potential theories for the role of the proteasome/aggresomes in the mechanisms of disease are discussed more fully in Section III.

4.2.7. Huntington's Disease: Expanded CAG Region of Huntingtin

Huntington's Disease is an inherited disorder characterized by progressive degeneration of the striatum and cerebral cortex (Harper, 1991). The expansion of exon 1 in the IT-15 gene by repeats of CAG sequence result in the expression of an expanded Huntingtin (HTT) protein containing repeats of glutamine from 38–182 residues (Ambrose *et al.*, 1994). Normally the HTT protein contains 8–37 glutamine residues, suggesting the toxicity of this defect resides in the expanded repeat region. The expanded HTT aggregates readily, while the normal 8–37 repeat HTT does not aggregate (Scherzinger *et al.*, 1999). Cell culture models (Lunkes and Mandel, 1998) and transgenic mice (<http://www.hdfoundation.org/workshop/200003HDMiceTable.pdf>) have also demonstrated that the expanded alleles of HTT readily form aggregates *in vivo*, while the normal (nonexpanded) forms do not aggregate. HTT can form both nuclear and cytoplasmic inclusions in both yeast and mammalian cells (Lunkes and Mandel, 1998; Muchowski *et al.*, 2002). There is evidence that the nuclear versus cytoplasmic localization of HTT may be cell cycle dependent (Martin-Aparicio *et al.*, 2002), and therefore, may be different in dividing and nondividing cells. HTT inclusions have been shown to be aggresomes, in the sense that they are formed via transport on MTs (Muchowski *et al.*, 2002; Taylor *et al.*, 2003). PolyQ inclusions in the cytoplasm form at the centrosome, and they are associated with rearranged vimentin (Waelter *et al.*, 2001). Expanded HTT protein is degraded by proteasomes, and the inclusions of expanded HTT protein contain Ub (Taylor *et al.*, 2003), proteasome subunits (Waelter *et al.*, 2001), chaperones, 14-3-3, and α -synuclein (Waelter *et al.*, 2001). These studies have also demonstrated an enhanced toxicity resulting from conditions that prevent aggresome formation (Muchowski *et al.*, 2002; Taylor *et al.*, 2003) suggesting that dispersed microaggregates are more harmful to cells than the formation of an aggresome—at least in the time frame of the described studies. There has been one report of MT-independent formation of HTT aggresomes (Meriin *et al.*, 2002), although in this report it was unclear if the disruption of MTs occurred after the aggresomes had already formed. It has already been suggested (Johnston *et al.*, 1998) that MT disruption after aggresome formation does not disperse the aggresomes into microaggregates. MTs provide an active transport for aggregated protein, not a force that maintains the aggresome at the centrosome. Trans-

genic mice inducible for the expression of an expanded HTT (Martin-Aparicio *et al.*, 2001) containing early HTT aggregates were shown to be degraded by the proteasome pathway. Therefore, mechanisms that degrade mutant HTT in cell culture experiments are the same mechanisms operating *in vivo*. Transient expression of the HTT transgene revealed that, in the presence of proteasome inhibitors the HTT protein did not disappear, whereas in the absence of proteasome inhibitors the HTT protein aggregates were effectively removed. This suggests there is a point at which the HTT aggregates can be removed prior to aggresome formation. (It is interesting to note that I have not yet seen aggresomes of CFTR or SOD1 disappear after they have been formed, even after cyclohexamide treatment; J.A. Johnston, unpublished observations). Behavioral analysis of animals during the transient HTT expression/suppression experiments revealed that the formation of HTT aggregates preceded the onset of motor impairment as measured by rotorod experiments (Martin-Aparicio *et al.*, 2001). At 12 weeks of HTT expression, the animals have visible aggregates, and behavior defects at which time the transgene expression was turned off. The aggregates were cleared after 3 weeks, and although the recovery of normal motor behavior was delayed, full recovery was evident at 8 weeks post-HTT suppression. Although there was a clear reduction in the size of the striatum in these animals, there was no measurable cell loss, leading to the suggestion that the aggregates themselves were not toxic to cells (Yamamoto *et al.*, 2000). This conclusion was supported by the use of neuronal cultures from the same animals, where the expression of the transgene did not lead to cell death after 7 days (Martin-Aparicio *et al.*, 2001). Although this may in fact be the case, the behavioral data clearly correlate the formation of aggregates to a loss of motor coordination, which if the transgene is continued to express, leads to greater symptomology (Yamamoto *et al.*, 2000; Martin-Aparicio *et al.*, 2001). As discussed in Section III, it may be that the toxicity of HTT protein is not from the aggregation per se, but on the neurotoxicity that is created from the competition for proteasome access by other proteasome substrates, and resulting neuronal dysfunction. The underlying molecular basis of this reversibility may be in the nature of the toxicity of the PolyQ region. Although it is not fully clear whether the structure of the expanded PolyQ region forms a hydrogen-bonded β -sheet (Perutz *et al.*, 1994) or a linear lattice (Bennett *et al.*, 2002), or a distribution of both, there is clearly a form that can be subject to removal even after initial aggregation (Martin-Aparicio *et al.*, 2001; Yamamoto *et al.*, 2000). It maybe important to identify this form of expanded PolyQ, as it may identify early pathology, as well as potential intervention points for therapeutics.

4.2.8. Charcot-Marie-Tooth Disease: Peripheral Myelin Protein 22 (PMP22)

Demyelinating peripheral neuropathies have been linked to duplications and mutations in the PMP22 gene (Lupski and Garcia, 2001). Like many of the proteins that comprise the neuronal insulating material myelin, PMP22 is a multipass transmembrane domain protein that is important in the formation of compact myelin (Pareek *et al.*, 1997). Similar to other membrane proteins discussed in this section, the synthesis and trafficking of the PMP22 occur through the secretory pathway, and is subject to quality control mechanisms to ensure the delivery of only functional protein to the cell surface (Pareek *et al.*, 1997). Two mutations in PMP22, Tr and TrJ, have defects in trafficking to the cell surface and are prone to aggregate (Tobler *et al.*, 2002). PMP22 is also similar to the CFTR protein and Cx50, in that a large proportion of the wild-type protein undergoes retrotranslocation and ERAD as a part of normal metabolism (Ryan *et al.*, 2002). The degradation rate of wild type, Tr, and TrJ mutants of PMP22 were all decreased significantly by the addition of proteasome inhibitors (Ryan *et al.*, 2002) in transfected rat Schwann cells. Immunofluorescence experiments also demonstrated that the addition of proteasome inhibitors resulted in the formation of Ub-positive, IF-associated, and MT-dependent pericentrosomal inclusions of insoluble PMP22 protein. Finally, transgenic mice expressing Tr/TrJ mutants of PMP22 have been shown to contain PMP22 and Ub-positive

intracellular inclusions (Ryan *et al.*, 2002). Wild-type mice contain few PMP22/Ub inclusions, although the heterozygotes have an increased number, and finally, the homozygous Tr/TrJ mice demonstrate pronounced pericentrosomal aggresomes as well as peripheral aggregates in Schwann cell processes. The PMP22 aggresomes were also shown to be colocalized with HSP70 and $\alpha\beta$ -crystallin. Similar to the mutant connexin50, the mutant TrJ (in heterozygous animals) can prevent the wtPMP22 from delivery to the cell surface (Tobler *et al.*, 2002). Distinct from the mutant connexins is the fact that the Tr/TrJ mutations also form aggresomes. In both cases, trafficking defects, and the cellular response to those defects results in pathology. Moreover, the fact that the wild-type protein can form aggresomes suggests that some sporadic disease may arise from conditions that result in decreased effectiveness of the ERAD pathway.

4.2.9. Cataracts: Connexin 50, 43

Gap junction proteins play a crucial role in the ability of cells to communicate and allow transfer of molecules between cells (Goodenough, 1992; Goodenough *et al.*, 1996). Gap junction proteins form intercellular channels that are composed of oligomeric assemblies of gap junction proteins called connexins (Cx). The Cxs are synthesized and processed through the secretory pathway to arrive at their residence in the plasma membrane. Mutations in the Cx50 gene have been linked to the formation of cataracts in humans (Shiels *et al.*, 1998; Berry *et al.*, 1999; Polyakov *et al.*, 2001). The degradation of Cx50 has been shown to be through ERAD mechanisms (Berthoud *et al.*, 2003). Characteristic of normal proteins that are difficult to fold, a proportion of the normal Cx undergoes retrotranslocation from the ER and degradation by cytoplasmic proteasomes. Moreover, a disruption in proteasome function results in the accumulation of Cx50 in aggresomes that colocalize with vimentin, require MTs for formation and are composed of insoluble protein (Berthoud *et al.*, 2003). In this case the authors suggested that cellular stress could provoke a disruption in the normal delivery of functional Cx to the plasma membrane and disrupt gap junction formation, preventing critical communication between lens fiber cells and lens epithelial cells (Berthoud *et al.*, 2003). Interestingly, the presence of cytosolic stress lead to an increase in the delivery of Cx32 to the cell surface, while ER-associated stress did not (VanSlyke and Musil, 2002). Similar results were obtained for Cx50 (Musil *et al.*, 2000). This suggests that the ability to increase intercellular communication via gap junctions be increased during cytosolic stress. Disruption of the communication between lens cell types via disruption of Cxs has been demonstrated to result in the formation of cataracts in mice (Gong *et al.*, 1997; White *et al.*, 1998). Interestingly, the human autosomal dominant mutation Cx50, hCx50P88S (Pal *et al.*, 1999) displays a trafficking defect when expressed in many cell types; however, this mutant does not result in the formation of aggresomes in cells (Pal *et al.*, 1999). Rather, the hCx50P88S is unable to be dislocated from the ER membrane to allow normal clearance by proteasomes. As a consequence, the mutant accumulates in the ER and causes a proliferation of membrane stacks, whereby little, if any, functional hCx50P88S arrive at the cell surface. The expression of the mutant hCx50P88S was shown to decrease the levels of normal, functional Cx50 at the cell surface and it may be that the mutant Cx50 coassembles into oligomeric complexes in the ER, but the folding defect of the mutant Cxs prevents the movement of the assembled complex past the ER. In this regard, the hCx50P88S, although not forming aggresomes as a means of toxicity, creates a disruption in cellular processes because it is refractory to the normal mechanisms that allow clearance and regulation of assembly of oligomeric gap junction complexes in the ER. The demonstration that normal Cx50 does form aggresomes suggests a mechanism whereby a disruption in the normal cellular processes could lead to an alteration in Cx50 degradation resulting in a change in the numbers of functional gap junction protein complexes at the cell surface altering intercellular communication, and allowing for the formation of cataracts in lens tissue.

4.2.10. Parkinson's Disease: Lewy Bodies/Parkin/ α -Synuclein

Parkinson's disease is characterized as a progressive loss of dopaminergic innervation of the striatum due to loss of dopaminergic neurons in the substantia nigra (Korlipara and Schapira, 2002). Pathologically, the loss of striatal innervation and decrease in dopamine levels is also accompanied by the presence of Lewy bodies in the cytoplasm of surviving neurons. This review will focus *only* on the data regarding Lewy Bodies and aggresomes. However, the role of the proteasome in PD may involve more than aggresome formation, and review articles are available for more information (Dauer and Przedborski, 2003). Lewy bodies were initially characterized and identified by Ub immunocytochemistry (Kuzuhara *et al.*, 1988). Recent human genetic data suggests PD may have a genetic component (Gasser, 2001), and the proteins identified in human genetic studies appear to have a role in the proteasome pathway. The identification of the α -synuclein gene in familial genetic studies of PD (Polymeropoulos *et al.*, 1997) lead to the discovery that the Lewy bodies are also α -synuclein positive (Spillantini *et al.*, 1997). In studies of isolated Lewy bodies, it has been shown that they are consistently composed of α -synuclein, Ub, and lipids (Gai *et al.*, 2000). Although cdk5 (Brion and Couck, 1995), proteasomes (Kwak *et al.*, 1991), Dorfin E3 ligase (Hishikawa *et al.*, 2003), Parkin E3 ligase (Schlossmacher *et al.*, 2002), and HDAC6 (Kawaguchi *et al.*, 2003) have also been reported as staining a large percentage of Lewy Bodies in pathological tissue. It has been suggested that Lewy bodies are aggresomes based on the immunochemical similarities (McNaught *et al.*, 2002c); however, the data providing a mechanistic correlation between aggresomes and Lewy Bodies is more compelling (Kawaguchi *et al.*, 2003). Experimental validation of proteasome impairment and aggresome formation in PD has been reported in pharmacological experiments *in vivo* in rodents. Specifically, the injection of proteasome inhibitor into the substantia nigra (McNaught *et al.*, 2002a) or the striatum (Fornai *et al.*, 2003) resulted in the loss of dopaminergic neurons and the remaining neurons contained Ub- and α -synuclein-positive inclusions. The presence of dopamine in the nigral neurons was a factor in the cell loss and inclusion formation (Fornai *et al.*, 2003). Interestingly, the isolation procedure for Lewy bodies is remarkably similar to the independently conceived isolation of aggresomes (Johnston *et al.*, 1998; Gai *et al.*, 1999). The available data suggests that the mechanisms underlying aggresome formation are also functioning to generate Lewy Bodies in PD.

Because of the obvious similarities of Lewy bodies and aggresomes, researchers have been involved in establishing the relationship of proteins found in Lewy bodies to the process of inclusion formation (aggresomes) in cells. At present, although probably unrecognized generally by the field of Parkinson's researchers, the data suggesting Parkin plays a major role in Lewy Body formation far outweighs the data suggesting α -synuclein as a central mediator of Lewy Body formation. Both α -synuclein and Parkin have been reported to form aggresomes in cells; however, the proportion of cells forming α -synuclein aggresomes in cells is extremely small and insufficient to perform most of the experiments to define the aggresome correlates (i.e., MT dependent formation, vimentin rearrangement, centrosome localization) with certainty. α -Synuclein forms aggregates after proteasome inhibition (Rideout *et al.*, 2001), dopamine exposure (Xu *et al.*, 2002), nitrative insult (Paxinou *et al.*, 2001; Hyun *et al.*, 2003), mitochondrial stress (Lee *et al.*, 2002; Trimmer *et al.*, 2004), overexpression with other proteins (Waelter *et al.*, 2001; Tanaka *et al.*, 2004), and as a result of human genetic mutation (Ostrerova-Golts *et al.*, 2000). Parkin forms aggresomes after overexpression, genetic mutation, proteasome inhibition (McNaught *et al.*, 2002c; Ardley *et al.*, 2003; Corti *et al.*, 2003; Gu *et al.*, 2003; Kawaguchi *et al.*, 2003; Tanaka *et al.*, 2004). There is controversy as to whether α -synuclein is ubiquitinated (Imai *et al.*, 2000; Sampathu *et al.*, 2003; Tofaris *et al.*, 2003), short-lived, or even degraded by proteasomes (Bennett *et al.*, 1999; Biasini *et al.*, 2004). From most of the reported studies, it is clear that α -synuclein and α -synuclein containing the human genetic mutations does not readily aggregate in cells. For example, nitrating conditions to induced 1–3% of α -synuclein

expressing cells to form aggregates (Paxinou *et al.*, 2001). This data revealed that at best 97% (and at worst 99%) of the cells did not respond, suggesting a relatively weak propensity to aggregate, especially when compared to truly aggregation prone proteins like CAG repeat, prion, connexins, etc., as discussed above, that readily aggregate after increased expression. After the overexpression of Parkin and synphilin, α -synuclein could be detected in aggresomes (Tanaka *et al.*, 2004), and α -synuclein has also been reported to associate with CAG repeat aggresomes (Waelter *et al.*, 2001); however, both of these studies suggest that the aggresome-forming process is not solely due to α -synuclein. Even at extremely high expression levels where α -synuclein is driven into inclusion bodies (in a process that may be independent of aggresome formation), it is not clear that the viral delivery vector has not affected the inclusion forming process in the cells (Lee and Lee, 2002) (see Virus Factories, Section II). Because α -synuclein is consistently found in Lewy Bodies, and has been demonstrated to be a component of the fibrils (Gai *et al.*, 2003) in Lewy bodies it is important to determine its involvement in the disease process. However, currently this is not at all clear, except that α -synuclein does not itself readily form aggresomes in cells. The structure of α -synuclein does not appreciably change due to the reported human mutations (Li *et al.*, 2001), although the changes may disrupt the interaction of α -synuclein with lipids (Davidson *et al.*, 1998; Eliezer *et al.*, 2001; Narayanan and Scarlata, 2001; Sharon *et al.*, 2001). Interestingly, the association of Rps5 with lipids promotes the ubiquitination of endocytic machinery and helps to regulate the delivery of cargo to intracellular membrane compartments (Dunn, 2004). Although α -synuclein does not yet have a clear function in cells, one way to link α -synuclein to the ubiquitination pathway (and therefore suggest an indirect yet crucial link to aggresome formation) could be that one part of α -synuclein serves as an adapter protein to link endocytic/exocytotic cargo to the membrane, and another domain promotes an association with Ub ligases like Rps5. As discussed in Section III, the role of the proteasome in the regulation of cell surface proteins in neurons is just recently becoming elucidated.

Interestingly, Parkin has clearly been demonstrated to form aggresomes in cells, and these aggresomes are formed via MT-dependent mechanisms, are associated with rearranged vimentin, and are Ub positive (McNaught *et al.*, 2002c; Ardley *et al.*, 2003; Corti *et al.*, 2003; Gu *et al.*, 2003; Kawaguchi *et al.*, 2003; Tanaka *et al.*, 2004). Most of the cells expressing Parkin in these studies demonstrated obvious aggresomes, providing a clearer relationship to aggresome formation than α -synuclein. Parkin is generally a short-lived protein, and has been shown to be induced after stress conditions (Imai *et al.*, 2000). Like α -synuclein, Parkin is a component of Lewy Bodies as assayed by both immunohistochemistry and immunoblotting techniques (Schlossmacher *et al.*, 2002), and its propensity to aggregate in cells suggests it resides in Lewy bodies due to accumulation as a misfolded protein. Based purely on the growing number of reports of Parkin aggresomes in the literature, and the reproducibility of the aggresomes that form from wild-type and mutant forms in the absence of exogenous stresses (unlike α -synuclein, which appears to require massive stress to aggregate), it is becoming clear that Parkin misfolding and aggregation could play a key role in sporadic disease. Previously, it was thought that because autosomal recessive juvenile PD patients (AR-JP) did not have Lewy bodies, that Parkin-related parkinsonian disorders may reflect a different disease process that affects dopaminergic neurons than those pathologies that result in Lewy bodies (Hattori *et al.*, 2000). Parkin is an E3 ligase (Shimura *et al.*, 2000; Imai *et al.*, 2002; Staropoli *et al.*, 2003), and the recessive nature of identified mutants (Bonifati *et al.*, 2001) suggests that inability to remove a crucial protein(s) due to lack of Parkin activity results in dopaminergic cell loss. However, this data could also be interpreted to mean that Parkin has a central role in the formation of Lewy bodies, and that in the absence of this functional role, juvenile onset disease is the result. This hypothesis is predilected on the idea that Lewy Bodies could be protective. Importantly, although most human genetic studies

have described Parkin as a recessive loss of function (Kitada *et al.*, 1998), new studies suggest that certain heterozygous mutations in Parkin can be linked to later onset “sporadic” disease (Foroud *et al.*, 2003). This suggests that these dominant/risk factor mutations may be acting through a misfolding and aggregation pathway that is characteristic of the other diseases discussed in this section. Of course, these later onset mutations could also be a result of haplo-insufficiency, as protein aggregation can also lead to loss of function, and we know that loss of Parkin function (in homozygous humans) leads to PD. Regardless of mechanism of the Parkin-linked later onset disease, therapeutic strategies that prevent loss of Parkin activity would probably be beneficial. Parkin also contains a Ub-like domain (UBL) at its N-terminus that is important for its function as a potential proteasome interacting motif (Sakata *et al.*, 2003; Upadhy and Hegde, 2003). Parkin activity can be regulated by proteolysis that may be related to neuroinflammation (Kahns *et al.*, 2003), and the inactivating cleavage of Parkin coincides with removal of the UBL from Parkin. It will be interesting to establish if the Parkin that accumulates in Lewy bodies is full length or UBL removed, as this information may offer clues to pathogenesis of PD, or biogenesis of Lewy bodies. This also suggests that environmental mechanisms may easily lead to a change in Parkin structure that not only results in loss of function, but also creates a misfolded protein aggregate in the cells, offering an important clue to sporadic PD.

5. Mechanisms of Aggresome Formation

Aside from the cellular practicalities of moving undegradable, aggregated protein from one place to another (see Section I, 3.4), an understanding of mechanisms leading to aggresome formation may provide the earliest clues to the development of pathological conditions. Moreover, we may also gain insight into the temporal sequence of events in aggresome formation: the onset of pathology and identification of potential intervention points as well as where optimal intervention/prevention points may exist. In this part of the review, we will discuss important concepts in three major areas that offer insight into mechanisms of aggresome formation. A thorough understanding of the role of proteasome biology, cellular neurobiology, and the role of the proteasome in neurons, and finally the biophysical process of fibril formation may ultimately provide a full understanding of mechanisms leading to the accumulation of misfolded protein in cells. Although these three areas themselves are vast and the literature immense, a focus on information that provides an intersection of these diverse areas can offer a unique insight into aggresome formation.

5.1. Proteasome Biology: Substrate Competition

Proteasomes are responsible for the degradation of most cellular proteins, in one way or another (**Figure 9-1; see color insert**; see Section I, 3.1) The proteasome has enormous capacity for substrates, and even the overexpression of a proteasome substrate in cells does not lead to a quantifiable change in degradative capacity (Dantuma *et al.*, 2000; Shimura *et al.*, 2000), and this suggests the proteasome can accommodate a large increase in load. However, in these same studies, the overexpression of a proteasome substrate containing a point mutation lead to a measurable decrease in proteasome degradation, and this decrease in proteolysis was significant enough to cause the accumulation of other proteasome substrates (Dantuma *et al.*, 2000; Bence *et al.*, 2001). The activity of the resident ATPases of the proteasome are efficient at unfolding globular substrates (Navon and Goldberg, 2001) but may require additional rounds of ATPase activity for malfolded proteins. Clearly, in pathological

tissue, the substrate recognition mechanisms are intact as many disease-linked proteins accumulate as ubiquitinated proteins. This data suggests pathology occurs through a defect in the proteasome, either unfolding or degradation. Conversely, the problem may lie with the substrate, in that effective targeting takes place but the protein is sufficiently aggregated that the fully functional proteasomes cannot unfold the protein to feed it into the catalytic channel. It may be that cellular proteasomes are subject to inactivation through changes in the cellular environment, as oxidation can inactivate the catalytic centers of the proteasome (Bulteau *et al.*, 2001). Alternatively, proteasomes may decrease in activity throughout the aging process (Sitte *et al.*, 2000a, 2000b; Carrard *et al.*, 2002; Gray *et al.*, 2003). Defects in mitochondria may result in depletion/limitation of cellular ATP, and prevent the ATP-intensive proteasome pathway from functioning properly to the point that protein accumulates and aggresomes form.

Although there are mechanisms to disrupt proteasome function through aging and damage, aggresome formation is most likely the result of substrate competition for access to the catalytic centers of the proteasome. It is difficult to reconcile the cell-type specific degeneration observed in many diseases with a general catalytic inactivation of proteasomes. It has been suggested that subpopulations of neurons are more susceptible to proteasome inactivation (McNaught *et al.*, 2002b) than others, although this does not explain the many diseases that manifest inclusion bodies in many different cell types. The hypothesis of proteasome competition posits that a bottleneck for a crucial parameter can create a decrease in the rate of access for substrate into the catalytic center of the proteasome. Access to, and degradation by, the proteasome encompasses all of the aspects of targeting, ubiquitination, and unfolding, and competition could occur at any of these steps. In this model, the proteasome is working at its normal high efficiency/rate; however, it is never able to degrade all of the substrates that need immediate turnover. This competition for proteasome access increases the transit time from substrate targeting to peptide backbone cleavage. This delay offers the substrate additional opportunity to adopt a conformation that may render it immutable to degradation. Moreover, the hydrophobic patch on the surface of the multi-Ub ladder could create a situation where a protein that does not aggregate through self-association, now aggregates through the aggregation of hydrophobic multi-Ub ladders.

Mechanistically, how can a protein aggregate impair proteasome function? As an example, consider the CAG repeat/polyglutamine proteins (also discussed in Section II, Huntington's Disease). The expanded stretch of polyglutamine residues has been proposed to lead to pathology through a few mechanisms, two of which are: (1) the adoption of the β -pleated sheet conformation, that leads to hydrogen bonding between antiparallel strands (Perutz *et al.*, 1994); (2) the formation of a linear lattice, that creates a multivalent interaction domain for ligand (Bennett *et al.*, 2002). Although it is still unclear which model more closely represents the cellular condition for aggregation, both models are consistent with a change that can cause a logjam at the proteasome. In the first mechanism, the hydrogen bonding between antiparallel sheets can result in increased residence time on the surface of the proteasome, as the resident ATPases unsuccessfully attempt to thread the protein into the mouth of the proteasome. In the second model, it may be that the multivalent interaction of ligand with the expanded PolyQ is sufficiently strong that, again, the ATPases are unable to thread the protein into the proteasome. It has been shown that a protein tightly bound to ligand is unable to be unfolded and degraded by the proteasome, even though the targeting of the protein has occurred normally as evidenced by accumulation of ubiquitinated substrate (Johnston *et al.*, 1995; Lee *et al.*, 2001). It is interesting to note that in both of these models the decrease in proteasome degradation has nothing to do with the actual catalytic centers of the proteasome; the decrease is almost completely attributable to the inability of the proteasome to thread an unfolded polypeptide chain into the mouth of the proteasome. Therefore, an isolation of proteasomes from these cells will likely yield proteasomes that are as active (in an *in vitro* activity assay) as proteasomes from unstressed cells, even

though the cellular defect is completely due to decreased proteasome activity. One recent report suggests that proteasome activity does decrease with age in a Huntington's disease transgenic mouse model (Zhou *et al.*, 2003); however, this study utilized crude cellular extract for the *in vitro* assay, and the aggregated HTT in the extract may still be able to compete for proteasome activity. This type of competition has been reported for test proteins in proteasome degradation assays (Lam *et al.*, 2000). The use of the GFP-degron transgenic animals (Lindsten *et al.*, 2003) will help to elucidate proteasome activity *in vivo* as a function of expression of disease-linked proteins like Huntingtin.

Because of the increased resident time of unfoldable substrate on the proteasome, it is likely that other substrates become delayed in their degradation. Although this delay may compound the aggregated protein problem, it is also clear that many cellular regulatory proteins rely on the proteasome for elimination of their biochemical activity, and a delay in their degradation can lead to profound alterations in cellular regulation and function. For example, the timely degradation of cyclin proteins is required for passage through specific stages in the cell cycle (Koepp *et al.*, 1999). A delay in degradation could lead to inappropriately activated cyclin and dysregulated phosphorylation of its substrates. It is likely that many cellular kinases are regulated in this fashion, and cyclin-dependent kinase5 (CDK5) (Kesavapany *et al.*, 2004) and serum-induced kinase (SNK) (Pak and Sheng, 2003) are specific neuronal kinases whose dysregulation can have profound consequences for neurons.

The most likely scenario for proteasome competition that results in aggresome formation is that proteins compete for access into the proteasome and the proteasome catalytic activity is not altered. Recently, two groups have described reporter systems to test this idea (Dantuma *et al.*, 2000; Bence *et al.*, 2001) in cells, and in animals (Lindsten *et al.*, 2003). Both of these models rely on a degradation signal fused to GFP, rendering the GFP short-lived and degraded by proteasomes. Overexpression of misfolded protein increases GFP fluorescence. Importantly, it was shown that simply overexpressing a proteasome substrate is not enough to cause proteasome inhibition and aggresome formation—the crucial factor is a protein that is misfolded. Presumably, a soluble globular folded protein is degraded in a timely fashion, as it does not require extra time for unfolding a low energy state conformation. However, the introduction of protein that misfolds results in the generation of aggresomes of not only itself, but of the test substrate also (Bence *et al.*, 2001). The aggregation of test substrate is curious, because in both cases the test sub is a GFP fused to a small degradation signal at either the N-terminus (Dantuma *et al.*, 2000) or the C-terminus (Bence *et al.*, 2001). GFP is known to be a well-folded and stable protein, and moreover, in both experimental systems, the aggregates of GFP test substrate were still fluorescent, indicating the GFP moiety was still properly folded. It may be that the multi-Ub chain and the hydrophobic patch on the surface of a multi-Ub chain create the propensity to form aggregates through interaction of the Ub chains. This provides yet another piece of evidence that direct coupling of targeting and degradation by proteasomes is temporally important, and delays in this (dis)assembly line can lead to problems for the intracellular homeostasis. The formation of aggresomes provides a mechanism for the removal of proteasome-clogging aggregates to allow for normal cellular function to continue.

Decreased proteasome activity is not lethal to cells, and other peptidases can be upregulated when the activity of the proteasome decreases (Wang *et al.*, 2000). Although this can delay the accumulation of ubiquitinated protein and allow cell survival (Wang *et al.*, 2000), the upregulation of cytoplasmic proteases to compensate for decrease in proteasome activity may have untoward consequences for cells in the long term.

The UBI4 Ub gene consists of four tandem repeats of the Ub sequence (Finley *et al.*, 1989; Monia *et al.*, 1990). Upon translation, each Ub moiety is cleaved as it emerges from the ribosome at the C-terminus to release a monomer Ub. A defect in the frame of Ub translation has been described

that results in an extension of the C-terminus of Ub. Specifically, transcription dinucleotide excision results in the extension of the C-terminus of Ub by 19 amino acids (van Leeuwen *et al.*, 2000). This extension results in a mutant form of Ub that can no longer form a multi-Ub ladder (because the C-terminus is not available for ϵ -amino linkage to substrate or other Ub in a multi-Ub chain); however, it has been shown to be recognized as a substrate for the Ub fusion degradation (UFD) pathway (Johnson *et al.*, 1992). The UFD pathway defines the conjugation of multi-Ub to Ub moieties that are present in Ub fusion proteins. The Ub+1 can then act as a cap on a multi-Ub chain and prevent UCH activity. Because the UCH cannot remove the Ub ladder the Ub+1 protein is not permitted entry into the proteasome (Lam *et al.*, 2000), although it is likely that it interacts with the proteasome through its multi-Ub ladder. Disruption of the channeling of unfolded substrates into the proteasome can lead to the inappropriate association of hydrophobic domains and increase the propensity of protein aggregation. Ub+1 can effectively compete for proteasome activity with other normal UPP substrates (Lam *et al.*, 2000), and delay the degradation of proteasome substrates *in vivo* (Lindsten *et al.*, 2002). Ub+1 protein has been identified in PSP, AD, and Mallory bodies (McPhaul *et al.*, 2002; Zatloukal *et al.*, 2002), and may therefore reflect a particular mechanism for the manifestation of aggresomes in pathological states. The fact that Ub+1 competes for proteasome activity suggests that proteasome competition can lead to aggresomes, and conversely, that aggresomes can arise from competition for proteasome degradation.

5.2. Cellular Neurobiology: The Role of the Ub–Proteasome Pathway in Neurons

Given the notion that proteasome competition underlies the accumulation of misfolded protein and aggresome formation, how is this concept related to the postmitotic function of neurons? Another way of asking this question: what neuronal functions rely on the Ub–proteasome pathway? In the last 2 years, there has been an explosion in our understanding of how the Ub–proteasome pathway is crucial for many events in the life of a neuron (fully reviewed in Hegde and DiAntonio, 2002; Steward and Schuman, 2003; Johnston and Madura, 2004): from the advance of the growth cone in development (Campbell and Holt, 2001), to the formation of a synapse at the target site (Hegde and DiAntonio, 2002), to the proper response to neurotransmitters leading to the alterations in synaptic architecture that form the basis of learning and memory (Colledge *et al.*, 2003). Genetic studies in *Drosophila* have identified Ub–proteasome enzymes as key molecules in the formation of specific neuronal structures. For example, nonstop (Poeck *et al.*, 2001) highwire (Hegde and DiAntonio, 2002), fat facets (Huang *et al.*, 1995), and Ariadne (Aguilera *et al.*, 2000) mutants form defective neuronal structures. A brief description of the emerging role of Ub in normal neuronal functions is followed by a discussion of how synaptic functions can become altered in disease, as well as how they may be exploited for early detection of potentially pathological conditions.

The growth cone during neuronal pathfinding in development is a complex process by which a growth cone of a neuron responds to extracellular cues that guide its movement to the appropriate destination (Tessier-Lavigne and Goodman, 1996). The growth cone can either be attracted to cues, or repulsed by them, and the response is based on the presence of particular cohorts of cell surface receptors (Harris and Holt, 1999). The proteasome pathway plays a crucial role in this process (Murphey and Godenschwege, 2002). Using *in vitro* primary neuronal culture models, proteasome inhibitors were shown to prevent the appropriate movement of a growth cone towards, or away from a particular cue (Campbell and Holt, 2001). Amazingly, the response was locally generated, in that isolated growth cones could move towards cues, suggesting functional autonomy from the cell body. Proteasome inhibitors prevent the response to extracellular cues in the isolated growth cones

(Campbell and Holt, 2001). These studies demonstrate that active proteasomes are required for cell surface protein modulation on growth cones in response to stimuli. Moreover, proteasome degradation does not take place exclusively at centrosomes, because isolated growth cones have been physically removed from the cell body.

After a neuronal growth cone has reached its target site for synapse formation, the Ub–proteasome pathway is involved in regulating the size of the synapse through Ubation and deubiquitination activities (Hegde and DiAntonio, 2002). The ubiquitination of specific proteins can serve to terminate signaling pathways. Specifically, Highwire is an E3 ligase in *Drosophila* that has been shown to ubiquitinate Medea in the BMP signaling pathway, and degradation of Medea serves to regulate synaptic size (McCabe *et al.*, 2004). Fat Facets is a deubiquitinating enzyme (homologue of UCH-L1 in mammals, whose role in learning and memory was identified in 1997) (Hegde *et al.*, 1997) that can partially complement the defects of highwire (DiAntonio *et al.*, 2001; Hegde and DiAntonio, 2002) suggesting that a balance between ubiquitination and deubiquitination is critical to regulate cell surface proteins and signaling pathways, as well as the developmental establishment of normal neuronal architecture.

In the last 2 years a central role for local protein synthesis and for degradation by the Ub–proteasome pathway in synaptic plasticity has begun to be identified (Steward and Schuman, 2003). Synaptic plasticity refers to the ability of the pre- and postsynaptic terminal to form stable connections, that are flexible enough to be removed or strengthened based on further experience. Long-term potentiation (LTP) refers to making synapses stronger (Eichenbaum, 1995; Huang *et al.*, 1996), and long-term depression (LTD) refers to making the synapses weaker (Linden and Connor, 1995; Linden, 1999), and these changes occur in response to neurotransmitter signals. In an elegant tour de force, it was demonstrated that both stimulation and inhibition of neuronal activity lead to the elimination of specific cohorts of proteins, and the stabilization of others (Ehlers, 2003). When these studies were repeated in isolated synaptosomes (the pre- and postsynaptic terminals associate tightly enough that they can be isolated from neuronal cell bodies intact) similar protein profiles were observed, demonstrating that a fully active proteasome pathway was present in synaptosomes, and is involved in the elimination and stabilization of synaptic protein in response to stimuli (Ehlers, 2003). Many of the proteins identified in this study were adapter proteins that are involved in the delivery and maintenance of cell surface proteins (Ehlers, 2003), and these types of proteins are increasingly being recognized as mediators of endocytosis through ubiquitination pathways (Hicke and Dunn, 2003). PSD95 is a major scaffold protein of the PSD, and is thought to function as an adapter protein that regulates cell surface proteins into and out of the dendrite membrane (Malinow and Malenka, 2002). Tetrodotoxin and Bicuculline did not change PSD95 levels, even though these pharmacological agents mimic inhibition and stimulation of neurons (Ehlers, 2003). However, specific activation of NMDA receptors was shown to induce the ubiquitination and degradation of PSD95 (Colledge *et al.*, 2003). The elimination of PSD95 was required for the loss of AMPA receptors after NMDA stimulation, and conversely, proteasome inhibitors prevented the decrease in AMPA receptors at the cell surface (Colledge *et al.*, 2003). This suggests that very specific stimuli can lead to the specific targeting of substrates to the proteasome, whose instability is not apparent under generic conditions. Proteasome inhibitors also prevented the development of LTD (Colledge *et al.*, 2003). LTD arises as a response to glutaminergic stimuli, and manifests at the molecular level by a decrease in AMPA receptors (Malinow and Malenka, 2002). The removal of PSD-95 by the proteasome allows for the proper response to glutaminergic stimuli, that is, the decrease in cell surface AMPA receptors. In the presence of proteasome inhibitors, PSD95 was not degraded, and the cell surface expression of AMPA receptors did not change in response to stimuli (Colledge *et al.*, 2003). The presence of excessive AMPA receptors can create excitotoxicity for neurons, suggesting that a functional proteasome

pathway is crucial for the maintenance and establishment of synaptic plasticity and response to stimuli. In addition, the removal of AMPA receptors likely requires a monoubiquitination event for targeting to the lysosome (Burbea *et al.*, 2002), implicating yet another ubiquitination dependent event in the normal regulation of synaptic plasticity.

For the synapse to respond to stimuli properly, the amount of neurotransmitter receptor at the cell surface needs to be regulated. In the case of the GABA_A receptor, it has been shown that the assembly of the multimer GABA receptor complex in the ER is not very efficient, with about 75% of immature subunits being turned over by ERAD mechanisms (Gorrie *et al.*, 1997), much like the CFTR protein (discussed in Section I). Recently, overexpression of a Ub binding protein, Plic-1, was shown to increase the amount of GABA receptor at the cell surface (Bedford *et al.*, 2001). The overexpression of Plic-1 probably works by preventing multiubiquitination of the GABA_A subunits, through the binding of the Ub association domain (UBA) of Plic-1 to a monoubiquitinated GABA_A subunit(s). This stabilization allows for receptor assembly and eventual delivery to the cell surface. Because neurotransmission relies on appropriate regulation of cell surface proteins, GABA receptors are probably not unique in their biosynthetic regulation. Opioid receptors (Petaja-Repo *et al.*, 2001), GPCRs (Bermak and Zhou, 2001), ion channels (Goodenough *et al.*, 1996; Musil *et al.*, 2000), and myelin (Ryan *et al.*, 2002) have clearly been shown to be regulated by ERAD. It is likely that neurons have an especially high burden on the ERAD machinery due to their enormous surface area, and dependence on the cell surface expression of complicated multipass transmembrane proteins.

Long-term synaptic changes related to memory involve new protein synthesis, and some of these new proteins are kinases that serve to remodel the PSD (Konietzko *et al.*, 1999). One recently characterized kinase, SNK, has been shown to phosphorylate SPAR, leading to SPAR degradation (Pak and Sheng, 2003). SPAR is a huge protein with many domains, and has been suggested as a major organizer of dendritic spine architecture as it can bind to actin, PSD95, Rap, and has a PDZ domain that may bind to a variety of cell surface proteins (Pak *et al.*, 2001). Overexpression of SPAR leads to enlargement of dendritic spine heads (Pak *et al.*, 2001), suggesting that a dysregulation of SPAR degradation can have serious consequences.

Neurons require a functional Ub–proteasome pathway for basic function like growth, synapse formation and maintenance, plasticity, and long-term memory. Defects the capacity of the proteasome or any of the enzymes involved in the targeting pathway could result in serious effects on neuronal function. The Ub–IF diseases are characterized by the presence of insoluble inclusions in affected cells. This review has discussed many of the ways that protein can end up in such an inclusion, and much of the data suggests that defects in degradation of misfolded proteins is a major contributor to the development of these diseases.

Our understanding of normal neuronal function and the role of the proteasome suggest that even subtle changes in proteasome degradation can have effects on neuropharmacology and neurotransmission. The concept of proteasome competition by misfolded protein has been discussed above. It is interesting to consider if there is any available data to suggest that early stages of disease be manifested by subtle alterations in synaptic response to pharmacological agents.

In fact, at least two examples, from the CAG repeat disorders, offer some interesting clues that support the notion that the earliest evidence of pathology may be long before the formation of obvious inclusions in cells.

As described for the SOD1 protein (Section II), IPCs form prior to the appearance of large cellular inclusions. IPCs also arise prior to early symptomology in transgenic animals for mutant SOD1 protein (Johnston *et al.*, 2000). IPCs probably also exist for many of the aggregation-prone proteins that cause disease as discussed in Section II. Misfolded proteins can create a competition for access to the proteasome, resulting in a delay of degradation of normal proteasome substrates (Bence *et al.*, 2001; Lindsten *et al.*, 2002). Do IPCs create competition for proteasome substrates that

can be evidenced as early alterations in normal synaptic function? Transgenic animals expressing the exon-1 of HTT with an expanded CAG repeat demonstrate age-dependent degeneration accompanied by the formation of large intracellular inclusions of HTT in the brain (Yamamoto *et al.*, 2000). Behavioral analysis of these animals revealed that the formation of aggregates preceded the onset of motor impairment as measured by rotorod experiments (Martin-Aparicio *et al.*, 2001). At 12 weeks of HTT expression, the animals have visible aggregates and behavior defects. Although there was a clear reduction in the size of the striatum in these animals, there was no measurable cell loss, leading to the suggestion that the aggregates themselves were not toxic to cells (Yamamoto *et al.*, 2000). This conclusion was supported by the use of neuronal cultures from the same animals, where the expression of the transgene did not lead to cell death after 7 days (Martin-Aparicio *et al.*, 2001). Although this may, in fact, be the case, the behavioral data clearly correlate the formation of aggregates to a loss of motor coordination, which if the transgene is continued to express, leads to greater symptomatology. Although both authors lament the lack of cell loss due to aggregate formation, the animal models may reveal that although aggregate formation is adaptive and therefore protective, the competition for Ub–proteasome pathway machinery eventually leads to cellular distress and dysfunction, as evidenced by robust behavioral phenotypes in animal models.

There is evidence for changes in neuronal function due to expression of expanded HTT in transgenic animals. A decrease in D1 receptors in the striatum of HTT symptomatic mice has been reported (Yamamoto *et al.*, 2000). D1 receptors are a GPCR, similar to the β -adrenergic receptors (b2R). The levels of b2R at the cell surface have been shown to be regulated by the proteasome pathway, in that the adapter molecule arrestin is degraded after recognition by Mdm2 ligase, and this allows clathrin coated pit endocytosis to remove b2R receptors from the cell surface (Shenoy *et al.*, 2001). In a remarkable set of experiments it has been shown that the inability to deubiquitinate the arrestin associated with the b2Rs results in the enhanced degradation of the b2Rs in lysosomes (Shenoy and Lefkowitz, 2003). If this mechanism is similar in the HTT animals, this suggests that the HTT, as it competes for proteasome degradation (Bence *et al.*, 2001), and deubiquitinating enzymes, can accelerate the degradation of D1 receptors via the competition at the level of ubiquitination/degradation. HTT has been shown to be an effective competitor of the proteasome, and the formation of inclusions, as well as overexpressed HTT substrate can effectively compete proteasome function resulting in impairment (Bence *et al.*, 2001). D1 receptor levels may depend on arrestin similar to other GPCRs (Shenoy *et al.*, 2001), although this has not yet been shown. It is consistent that the loss of D1 receptors at early stages in pathology in the HTT exon 1 transgenic mice is correlated to the accumulation of HTT protein as the HTT competes for proteasome degradation with adapter proteins that regulate D1 receptor at the cell surface. Alternatively, the biogenesis of D1 receptor and delivery to the plasma membrane may be affected by proteasome impairment, because the ER export of D1 receptors has been shown to be a crucial regulatory mechanism (Bermak and Zhou, 2001).

Pharmacological experiments may also reveal changes in other crucial cell surface molecules that are regulated by proteasome activity. For example, the number of AMPA GluRs at the cell surface is suggested to be regulated by ubiquitination of PSD95 (Colledge *et al.*, 2003), and specific pharmacological agents may reveal increases or decreases in AMPA receptors as a function of proteasome competition. In addition to PSD-95-dependent removal of AMPA from the cell surface, the delivery of new GluR to the cell surface from the biosynthetic machinery can be impaired by a disruption of normal subunit assembly mechanisms; for example, the NR2B subunit is degraded by proteasomes to regulate the number of receptors delivered to the cell surface (Huh and Wenthold, 1999). It may be that in conditions of proteasome impairment, the GluR1/NR2B is stabilized and results in more GluR delivery to the cell surface. Either the impaired degradation of PSD-95, or the increase in GluRs could result in excitotoxicity and sensitivity to glutaminergic agonists and antagonists.

In fact, recent studies in an HTT mouse demonstrated that the onset of pathological symptoms is correlated to an increase in sensitivity to excitotoxic stimuli, as well as agonists of GluRs (Zeron *et al.*, 2002). The effect of aggregated HTT on specific subtypes of receptors has been studied in tissue culture systems (Zeron *et al.*, 2001), and it would be interesting to use the proteasome activity GFP reporter cell lines (Dantuma *et al.*, 2000; Bence *et al.*, 2001) to determine if the pharmacological changes are coincident with an increase in GFP. Moreover, other studies with an HTT transgenic model have described resistance to quinolinic acid (Hansson *et al.*, 1999). Both of these different transgenic HTT models are consistent with the notion that the HTT protein has an effect on the Ub–proteasome pathway that impacts the normal regulation of cell surface proteins that can manifest in changes in neuropharmacology.

Interestingly, a focal loss of glutamate transporter EAAT2 has been reported to coincide with the appearance of symptoms in the SOD1 transgenic mouse (Howland *et al.*, 2002). It would be interesting to know if EAAT2 biosynthesis (i.e., ERAD) or turnover from the plasma membrane is regulated by the Ub–proteasome pathway. The accumulation of SOD1 IPCs may create competition for proteasome activity, and subsequently change EAAT2 levels in cells. In this case, the use of pharmacological agents may help to reveal the earliest time at which changes in EAAT2 appear. It would also be interesting to cross the SOD1 animals with the GFP–proteasome reporter animals (Lindsten *et al.*, 2003) to determine if IPCs correlate to changes in pharmacology and increased GFP.

In summary, the complex regulation of cell surface proteins in neurons that allows for appropriate neurotransmission depends on a functional Ub–proteasome pathway, and a few basic examples have been presented here. Factors that decrease the efficiency of targeting mechanisms and degradation capacity can have profound effects on neuronal function. These effects may be manifest much earlier than the appearance of large cellular inclusions, and insightful pharmacological experiments in transgenic animals may allow for early detection of pathology, as well as reveal novel intervention points. As demonstrated by the HTT transgenic models, the inclusions do not kill cells (Martin-Aparicio *et al.*, 2001), but the long-term effects of dysregulated neurotransmission may lead to cell loss.

Finally, a note on the location of aggresomes in neurons, and its relationship to the architecture of the neuron. The formation of a neuronal aggresome may occur at the centrosome, but may also be in an axon or dendrite (i.e., I suggest that dystrophic neurites and axonal spheroids may be aggresomes and formed from similar mechanisms as pericentrosomal aggresomes). As described in Section I, 3.3 and 3.4, the MT network of a neuron is discontinuous (Ahmad and Baas, 1995). The clearance of small aggregates from the dendrite may not result in the transportation of these aggregates all the way back to the cell body, but as an insoluble deposition into a portion of the dendrite that is less spatially constricted than the dendritic spines. Although the formation of pericentrosomal aggresomes in neurons may form from microaggregates collected from axons and dendrites, they could also arise from mechanisms related to transient aggresomes (Section II, 3.1). For example, prolonged proteolytic stress at a peripheral synapse may result in a signal to the cell body to suspend (at least temporarily) the delivery of protein(s) to the synapse. In this model the resulting aggresome would have formed from the protein that was produced in the cell body and never delivered to peripheral sites. Prolonged activation of such a stress pathway may ultimately lead to impairment in protein degradation regulation, leading to deposition of insoluble protein in a pericentrosomal location.

5.3. The Biophysical Process of Fibril Formation

The formation of protein fibrils is a generally a concentration-dependent biophysical process (Horwich, 2002). Some fibril-forming proteins have been selected for specific cellular func-

tions through evolution. Actin and tubulin form functional polymers in cells that perform vital functions such as structural organization, intracellular transport, and functional response to stimuli. The polymerization of soluble monomers into fibrils occurs through a nucleation by proteins that lower the critical concentration required for polymerization (Li *et al.*, 1995; Moon and Drubin, 1995; Desai and Mitchison, 1997). For example, γ -tubulin resides at the centrosome and serves as a nucleating protein for the polymerization of $\alpha\beta$ -tubulin into MTs. In the absence of γ -tubulin, the critical concentration for $\alpha\beta$ -tubulin to form MTs is much higher (Zheng *et al.*, 1998). The dynamic interplay between $\alpha\beta$ - and γ -tubulin proteins allows functional regulation over the assembly and disassembly of the MT polymer (Desai and Mitchison, 1997). Proteins associated with pathology also form fibrils, but these fibrils are not functional components of cells. These proteins are considered to form fibrils as part of an abnormal folding process. In this instance, the fibril forming process would be almost exclusively a concentration-dependent phenomenon, as the resulting fibrils do not have a functional role in cells and are only seen associated with pathological conditions. However, a common denominator for both of these fibril polymerization reactions is that the formation of fibrils is an energetically favorable process, and that the maintenance of soluble monomers/multimers is energetically less favorable. Thus, it may be that the ability to concentrate aggregated protein into one cellular location serves (at least) two purposes: (1) decrease the crowding in the cytoplasm, and (2) increase the local multimer concentration to allow for fibril formation. This particular interpretation of aggresomes would suggest that they serve as a protective mechanism for cells.

Aggresomes form as a cellular response to the accumulation of misfolded protein and are an insoluble Ub- and IF-positive inclusion in cells. The description of aggresomes provides an explanation for presence of homogeneous protein fibrils in cells, and suggests that cells attempt to mitigate the effects of misfolded proteins by a specific cellular pathway. Because this pathway concentrates misfolded protein in a particular area, the likelihood is increased for the critical concentration for protein fibrillation than would be expected from the limited diffusional accumulation of a single type of protein in one location in a cell. A stable homogeneous protein fibril is at a much lower energy state than a misfolded monomer/multimer of protein (Horwich, 2002). It may be important to consider that aggresomes are a protective mechanism for cells.

6. Future Directions

Are aggresomes protective or destructive? It will be interesting to know if the cells that are lost in Ub-IF degenerative disease were unable to make aggresomes. Because intracellular inclusions are a pathological hallmark of many degenerative diseases, it will be important to understand if the cells that remain and are observed in pathological analysis were protected because they could form an aggresome, or, if the first cells to form aggresomes were the earliest cells to succumb to pathology and degenerate, and the remaining cells formed aggresomes at a later stage in disease. Transgenic animals that closely model disease (Gurney, 2000) and animals that can serve as reporters of proteasome function (Lindsten *et al.*, 2003) can be combined to provide a temporal understanding of protein aggregation and the earliest signs of pathology. Moreover, as we learn more about the precise mechanisms regulating cell surface proteins in neurons we may be able to develop early diagnostic tools that correlate decreases in proteasome function/protein aggregation to changes in neuropharmacology. The role of the Ub-proteasome pathway in neurons is central to neuronal function, and understanding the normal regulation of proteins by this pathway may provide the most insightful clues to protein aggregation linked degenerative diseases.

7. Abbreviations

ALS	Amyotrophic lateral sclerosis
ASFV	African swine fever virus
CFTR	Cystic fibrosis transmembrane conductance regulator
CDK5	Cyclin dependent kinase 5
CJD	Crutzfeldt-Jacob disease
Cx	Connexins
DALIS	Dendritic cell aggresome-like structure
DC	Dendritic cells
DRM	Desmin related myopathy
DSG	15-Deoxyspergualin
EBNA-1	Epstein-Barr nuclear antigen-1
ER	Endoplasmic reticulum
ERAD	ER associated degradation
FFI	Fatal familial insomnia
FV3	Frog virus 3
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GluR1	Glutamate receptor
GSS	Gerstmann-Straussler-Scheinker
HD	Huntington's disease
HIF	Hypoxia inducible factor
HSP	Heat shock protein
HTT	Huntingtin
IF	Intermediate filament
IPC	Insoluble protein complex
MT	Microtubule
NF	Neurofilament
PD	Parkinson's disease
PMP22	Peripheral myelin protein 22
PPI	Peptidylprolyl isomerase
PrP	Prion protein
RP	Retinitis Pigmentosa
SCF	Skp-cullin-F box
SNK	serum induced kinase
SOD1	Superoxide dismutase
Ub	Ubiquitin
UBA	Ubiquitin-activating enzymes
UBC	Ubiquitin-conjugating enzyme
UCH	Ubiquitin C-terminal hydrolase

References

- Aguilera, M., Oliveros, M., Martinez-Padron, M., Barbas, J.A., and Ferrus, A. (2000). Ariadne-1: a vital *Drosophila* gene is required in development and defines a new conserved family of ring-finger proteins. *Genetics* 155:1231–1244.

- Ahmad, F.J., and Baas, P.W. (1995). Microtubules released from the neuronal centrosome are transported into the axon. *J. Cell Sci.* 108:2761–2769.
- Allen, S., Heath, P.R., Kirby, J., Wharton, S.B., Cookson, M.R., Menzies, F.M., Banks, R.E., and Shaw, P.J. (2003). Analysis of the cytosolic proteome in a cell culture model of familial amyotrophic lateral sclerosis reveals alterations to the proteasome, antioxidant defenses, and nitric oxide synthetic pathways. *J. Biol. Chem.* 278:6371–6383.
- Ambrose, C.M., Duyao, M.P., Barnes, G., Bates, G.P., Lin, C.S., Srinidhi, J., Baxendale, S., Hummerich, H., Lehrach, H., and Altherr, M. (1994). Structure and expression of the Huntington's disease gene: evidence against simple inactivation due to an expanded CAG repeat. *Somat. Cell Mol. Genet.* 20:27–38.
- Amerik, A., Swaminathan, S., Krantz, B.A., Wilkinson, K.D., and Hochstrasser, M. (1997). In vivo disassembly of free polyubiquitin chains by yeast Ubp14 modulates rates of protein degradation by the proteasome. *EMBO J.* 16:4826–4838.
- Andersen, S.S. (1999). Molecular characteristics of the centrosome. *Int. Rev. Cytol.* 187:51–109.
- Ardley, H.C., Scott, G.B., Rose, S.A., Tan, N.G., Markham, A.F., and Robinson, P.A. (2003). Inhibition of proteasomal activity causes inclusion formation in neuronal and non-neuronal cells overexpressing Parkin. *Mol. Biol. Cell* 14:4541–4556.
- Baas, P.W. (2002). Neuronal polarity: microtubules strike back [comment]. *Nat. Cell Biol.* 4:E194–E195.
- Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. *Nature* 392:245–252.
- Bardag-Gorce, F., Riley, N., Nguyen, V., Montgomery, R.O., French, B.A., Li, J., van Leeuwen, F.W., Lungu, W., McPhaul, L.W., and French, S.W. (2003). The mechanism of cytokeratin aggresome formation: the role of mutant ubiquitin (UBB+1). *Exp. Mol. Pathol.* 74:160–167.
- Bedford, F.K., Kittler, J.T., Muller, E., Thomas, P., Uren, J.M., Merlo, D., Wisden, W., Triller, A., Smart, T.G., and Moss, S.J. (2001). GABA(A) receptor cell surface number and subunit stability are regulated by the ubiquitin-like protein Plic-1. *Nat. Neurosci.* 4:908–916.
- Benaroudj, N., Zwickl, P., Seemuller, E., Baumeister, W., and Goldberg, A.L. (2003). ATP hydrolysis by the proteasome regulatory complex PAN serves multiple functions in protein degradation [see comment]. *Mol. Cell* 11:69–78.
- Bence, N.F., Sampat, R.M., and Kopito, R.R. (2001). Impairment of the ubiquitin–proteasome system by protein aggregation [see comment]. *Science* 292:1552–1555.
- Bennett, M.C., Bishop, Y.L., Chock, P.B., Chase, T.N., and Mouradian, M.M. (1999). Degradation of a-synuclein by proteasome. *J. Biol. Chem.* 274:33855–33858.
- Bennett, M.J., Huey-Tubman, K.E., Herr, A.B., West, A.P., Jr., Ross, S.A., and Bjorkman, P.J. (2002). Inaugural article: a linear lattice model for polyglutamine in CAG-expansion diseases. *Proc. Natl. Acad. Sci. USA* 99:11634–11639.
- Bermak, J.C., and Zhou, Q.Y. (2001). Accessory proteins in the biogenesis of G protein-coupled receptors. *Mol. Intervent.* 1:282–287.
- Berry, V., Mackay, D., Khaliq, S., Francis, P.J., Hameed, A., Anwar, K., Mehdi, S.Q., Newbold, R.J., Ionides, A., Shiels, A., Moore, T., and Bhattacharya, S.S. (1999). Connexin 50 mutation in a family with congenital “zonular nuclear” pulverulent cataract of Pakistani origin. *Hum. Genet.* 105:168–170.
- Berthoud, V.M., Minogue, P.J., Guo, J., Williamson, E.K., Xu, X., Ebihara, L., and Beyer, E.C. (2003). Loss of function and impaired degradation of a cataract-associated mutant connexin50. *Eur. J. Cell Biol.* 82:209–221.
- Biasini, E., Fioriti, L., Ceglia, I., Invernizzi, R., Bertoli, A., Chiesa, R., and Forloni, G. (2004). Proteasome inhibition and aggregation in Parkinson's disease: a comparative study in untransfected and transfected cells. *J. Neurochem.* 88:545–553.
- Bonifati, V., De Michele, G., Lucking, C.B., Durr, A., Fabrizio, E., Ambrosio, G., Vanacore, N., De Mari, M., Marconi, R., Capus, L., Breteler, M.M., Gasser, T., Oostra, B., Wood, N., Agid, Y., Filla, A., Meco, G., Brice, A., and Italian Pd Genetics Study Group, French PD Genetics Study Group and the European Consortium on genetic susceptibility in Parkinson's Disease. (2001). The parkin gene and its phenotype. *Neurol. Sci.* 22:51–52.
- Borchelt, D.R., Lee, M.K., Slunt, H.S., Guarnieri, M., Xu, Z.S., Wong, P.C., Brown, R.H., Jr., Price, D.L., Sisodia, S.S., and Cleveland, D.W. (1994). Superoxide dismutase 1 with mutations linked to familial amyotrophic lateral sclerosis possesses significant activity. *Proc. Natl. Acad. Sci. USA* 91:8292–8296.
- Borchelt, D.R., Guarnieri, M., Wong, P.C., Lee, M.K., Slunt, H.S., Xu, Z.S., Sisodia, S.S., Price, D.L., and Cleveland, D.W. (1995). Superoxide dismutase 1 subunits with mutations linked to familial amyotrophic lateral sclerosis do not affect wild-type subunit function. *J. Biol. Chem.* 270:3234–3238.
- Borges, K., and Dingledine, R. (1998). AMPA receptors: molecular and functional diversity. *Prog. Brain Res.* 116:153–170.
- Braun, B.C., Glickman, M., Kraft, R., Dahlmann, B., Kloetzel, P.M., Finley, D., and Schmidt, M. (1999). The base of the proteasome regulatory particle exhibits chaperone-like activity. *Nat. Cell Biol.* 1:221–226.

- Brion, J.P., and Couck, A.M. (1995). Cortical and brainstem-type Lewy bodies are immunoreactive for the cyclin-dependent kinase 5. *Am. J. Pathol.* 147:1465–1476.
- Brooks, P., Fuentes, G., Murray, R.Z., Bose, S., Knecht, E., Rechsteiner, M.C., Hendil, K.B., Tanaka, K., Dyson, J., and Rivett, J. (2000). Subcellular localization of proteasomes and their regulatory complexes in mammalian cells. *Biochem. J.* 346(Pt 1):155–161.
- Bruijn, L.I., and Cleveland, D.W. (1996). Mechanisms of selective motor neuron death in ALS: insights from transgenic mouse models of motor neuron disease. *Neuropathol. Appl. Neurobiol.* 22:373–387.
- Bulteau, A.L., Lundberg, K.C., Humphries, K.M., Sadek, H.A., Szveda, P.A., Friguet, B., and Szveda, L.I. (2001). Oxidative modification and inactivation of the proteasome during coronary occlusion/reperfusion. *J. Biol. Chem.* 276: 30057–30063.
- Burbea, M., Dreier, L., Dittman, J.S., Grunwald, M.E., and Kaplan, J.M. (2002). Ubiquitin and AP180 regulate the abundance of GLR-1 glutamate receptors at postsynaptic elements in *C. elegans*. *Neuron* 35:107–120.
- Burkhardt, J.K., Echeverri, C.J., Nilsson, T., and Vallee, R.B. (1997). Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. *J. Cell Biol.* 139: 469–484.
- Campbell, D.S., and Holt, C.E. (2001). Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation [see comment]. *Neuron* 32:1013–1026.
- Carrard, G., Bulteau, A.L., Petropoulos, I., and Friguet, B. (2002). Impairment of proteasome structure and function in aging. *Int. J. Biochem. Cell Biol.* 34:1461–1474.
- Chavez Zobel, A.T., Loranger, A., Marceau, N., Theriault, J.R., Lambert, H., and Landry, J. (2003). Distinct chaperone mechanisms can delay the formation of aggregates by the myopathy-causing R120G alphaB-crystallin mutant. *Hum. Mol. Genet.* 12:1609–1620.
- Chen, M., Goorha, R., and Murti, K.G. (1986). Interaction of frog virus 3 with the cytomatrix. IV. Phosphorylation of vimentin precedes the reorganization of intermediate filaments around the virus assembly sites. *J. Gen. Virol.* 67:915–922.
- Chou, Y.H., Ngai, K.L., and Goldman, R. (1991). The regulation of intermediate filament reorganization in mitosis. p34cdc2 phosphorylates vimentin at a unique N-terminal site. *J. Biol. Chem.* 266:7325–7328.
- Chou, Y.H., Khuon, S., Herrmann, H., and Goldman, R.D. (2003). Nestin promotes the phosphorylation-dependent disassembly of vimentin intermediate filaments during mitosis. *Mol. Biol. Cell* 14:1468–1478.
- Ciechanover, A. (1998). The ubiquitin–proteasome pathway: on protein death and cell life. *EMBO J.* 17:7151–7160.
- Cleveland, D.W., Bruijn, L.I., Wong, P.C., Marszalek, J.R., Vechio, J.D., Lee, M.K., Xu, X.S., Borchelt, D.R., Sisodia, S.S., and Price, D.L. (1996). Mechanisms of selective motor neuron death in transgenic mouse models of motor neuron disease. *Neurology* 47:S54–S61 [discussion S61–S62].
- Cohen, E., and Taraboulos, A. (2003). Scrapie-like prion protein accumulates in aggregates of cyclosporin A-treated cells. *EMBO J.* 22:404–417.
- Colledge, M., Snyder, E.M., Crozier, R.A., Soderling, J.A., Jin, Y., Langeberg, L.K., Lu, H., Bear, M.F., and Scott, J.D. (2003). Ubiquitination regulates PSD-95 degradation and AMPA receptor surface expression. *Neuron* 40: 595–607.
- Conaway, R.C., Brower, C.S., and Conaway, J.W. (2002). Emerging roles of ubiquitin in transcription regulation. *Science* 296:1254–1258.
- Corti, O., Hampe, C., Koutnikova, H., Darios, F., Jacquier, S., Prigent, A., Robinson, J.C., Pradier, L., Ruberg, M., Mirande, M., Hirsch, E., Rooney, T., Fournier, A., and Brice, A. (2003). The p38 subunit of the aminoacyl-tRNA synthetase complex is a Parkin substrate: linking protein biosynthesis and neurodegeneration. *Hum. Mol. Genet.* 12:1427–1437.
- Craig, K.L., and Tyers, M. (1999). The F-box: a new motif for ubiquitin dependent proteolysis in cell cycle regulation and signal transduction. *Prog. Biophys. Mol. Biol.* 72:299–328.
- Czar, M.J., Lyons, R.H., Welsh, M.J., Renoir, J.M., and Pratt, W.B. (1995). Evidence that the FK506-binding immunophilin heat shock protein 56 is required for trafficking of the glucocorticoid receptor from the cytoplasm to the nucleus. *Mol. Endocrinol.* 9:1549–1560.
- Dangond, F., Hwang, D., Camelo, S., Pasinelli, P., Frosch, M.P., Stephanopoulos, G., Brown, R.H., Jr., and Gullans, S.R. (2004). Molecular signature of late-stage human ALS revealed by expression profiling of postmortem spinal cord gray matter. *Physiol. Genom.* 16:229–239.
- Dantuma, N.P., Lindsten, K., Glas, R., Jellne, M., and Masucci, M.G. (2000). Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells [see comment]. *Nat. Biotechnol.* 18: 538–543.
- Dauer, W., and Przedborski, S. (2003). Parkinson's disease: mechanisms and models. *Neuron* 39:889–909.

- Davidson, W.S., Jonas, A., Clayton, D.F., and George, J.M. (1998). Stabilization of alpha-synuclein secondary structure upon binding to synthetic membranes. *J. Biol. Chem.* 273:9443–9449.
- Demand, J., Alberti, S., Patterson, C., and Hohfeld, J. (2001). Cooperation of a ubiquitin domain protein and an E3 ubiquitin ligase during chaperone/proteasome coupling [see comment]. *Curr. Biol.* 11:1569–1577.
- den Engelsman, J., Keijsers, V., de Jong, W.W., and Boelens, W.C. (2003). The small heat-shock protein alpha B-crystallin promotes FBX4-dependent ubiquitination. *J. Biol. Chem.* 278:4699–4704.
- Desai, A., and Mitchison, T.J. (1997). Microtubule polymerization dynamics. *Annu. Rev. Cell Dev. Biol.* 13:83–117.
- Deshaies, R.J. (1999). SCF and Cullin/ring H2-based ubiquitin ligases. *Annu. Rev. Cell Dev. Biol.* 15:435–467.
- Deveraux, Q., Ustrell, V., Pickart, C., and Rechsteiner, M. (1994). A 26S protease subunit that binds ubiquitin conjugates. *J. Biol. Chem.* 269:7059–7061.
- DiAntonio, A., Haghighi, A.P., Portman, S.L., Lee, J.D., Amaranto, A.M., and Goodman, C.S. (2001). Ubiquitination-dependent mechanisms regulate synaptic growth and function. *Nature* 412:449–452.
- Dingledine, R., Borges, K., Bowie, D., and Traynelis, S.F. (1999). The glutamate receptor ion channels. *Pharmacol. Rev.* 51:7–61.
- Dinodum, A., Harvey, K.F., Komwatana, P., Young, J.A., Kumar, S., and Cook, D.I. (1998). Nedd4 mediates control of an epithelial Na⁺ channel in salivary duct cells by cytosolic Na⁺. *Proc. Natl. Acad. Sci. USA* 95:7169–7173.
- Donaldson, K.M., Li, W., Ching, K.A., Batalov, S., Tsai, C.C., and Joazeiro, C.A. (2003). Ubiquitin-mediated sequestration of normal cellular proteins into polyglutamine aggregates. *Proc. Natl. Acad. Sci. USA* 100:8892–8897.
- Dryja, T.P., McGee, T.L., Reichel, E., Hahn, L.B., Cowley, G.S., Yandell, D.W., Sandberg, M.A., and Berson, E.L. (1990). A point mutation of the rhodopsin gene in one form of retinitis pigmentosa. *Nature* 343:364–366.
- Dryja, T.P., Hahn, L.B., Cowley, G.S., McGee, T.L., and Berson, E.L. (1991). Mutation spectrum of the rhodopsin gene among patients with autosomal dominant retinitis pigmentosa. *Proc. Natl. Acad. Sci. USA* 88:9370–9374.
- Dunn, R., Klos, D.A., Adler, A.S., and Hicke, L. (2004). The C2 domain of the Rsp5 ubiquitin ligase binds membrane phosphoinositides and directs ubiquitination of endosomal cargo. *J. Cell Biol.* 165:135–144.
- Ehlers, M.D. (2003). Activity level controls postsynaptic composition and signaling via the ubiquitin–proteasome system. *Nat. Neurosci.* 6:231–242.
- Eichenbaum, H. (1995). Spatial learning. The LTP-memory connection. *Nature* 378:131–132.
- Eliez, D., Kutluay, E., Bussell, R., Jr., and Browne, G. (2001). Conformational properties of alpha-synuclein in its free and lipid-associated states. *J. Mol. Biol.* 307:1061–1073.
- Fabunmi, R.P., Wigley, W.C., Thomas, P.J., and DeMartino, G.N. (2000). Activity and regulation of the centrosome-associated proteasome. *J. Biol. Chem.* 275:409–413.
- Fink, A.L. (1999). Chaperone-mediated protein folding. *Physiol. Rev.* 79:425–449.
- Finley, D., Bartel, B., and Varshavsky, A. (1989). The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. *Nature* 338:394–401.
- Flannery, J.G., Farber, D.B., Bird, A.C., and Bok, D. (1989). Degenerative changes in a retina affected with autosomal dominant retinitis pigmentosa. *Invest. Ophthalmol. Vis. Sci.* 30:191–211.
- Fornai, F., Lenzi, P., Gesi, M., Ferrucci, M., Lazzeri, G., Busceti, C.L., Ruffoli, R., Soldani, P., Ruggieri, S., Alessandri, M.G., and Paparelli, A. (2003). Fine structure and biochemical mechanisms underlying nigrostriatal inclusions and cell death after proteasome inhibition. *J. Neurosci.* 23:8955–8966.
- Foroud, T., Uniacke, S.K., Liu, L., Pankratz, N., Rudolph, A., Halter, C., Shults, C., Marder, K., Conneally, P.M., Nichols, W.C., and Parkinson Study Group. (2003). Heterozygosity for a mutation in the parkin gene leads to later onset Parkinson disease. *Neurology* 60:796–801.
- French, B.A., van Leeuwen, F., Riley, N.E., Yuan, Q.X., Bardag-Gorce, F., Gaal, K., Lue, Y.H., Marceau, N., and French, S.W. (2001). Aggresome formation in liver cells in response to different toxic mechanisms: role of the ubiquitin–proteasome pathway and the frameshift mutant of ubiquitin. *Exp. Mol. Pathol.* 71:241–246.
- Fuchs, S.Y., Spiegelman, V.S., and Kumar, K.G. (2004). The many faces of beta-TrCP E3 ubiquitin ligases: reflections in the magic mirror of cancer. *Oncogene* 23:2028–2036.
- Gai, W.P., Power, J.H., Blumbergs, P.C., Culvenor, J.G., and Jensen, P.H. (1999). Alpha-synuclein immunoprecipitation of glial inclusions from multiple system atrophy brain tissue reveals multiprotein components. *J. Neurochem.* 73:2093–2100.
- Gai, W.P., Yuan, H.X., Li, X.Q., Power, J.T., Blumbergs, P.C., and Jensen, P.H. (2000). In situ and in vitro study of colocalization and segregation of alpha-synuclein, ubiquitin, and lipids in Lewy bodies. *Exp. Neurol.* 166:324–333.
- Gai, W.P., Pountney, D.L., Power, J.H., Li, X.Q., Culvenor, J.G., McLean, C.A., Jensen, P.H., and Blumbergs, P.C. (2003). alpha-Synuclein fibrils constitute the central core of oligodendroglial inclusion filaments in multiple system atrophy. *Exp. Neurol.* 181:68–78.

- Galnigiana, M.D., Radanyi, C., Renoir, J.M., Housley, P.R., and Pratt, W.B. (2001). Evidence that the peptidylprolyl isomerase domain of the hsp90-binding immunophilin FKBP52 is involved in both dynein interaction and glucocorticoid receptor movement to the nucleus. *J. Biol. Chem.* 276:14884–14889.
- Ganesh, S., Delgado-Escueta, A.V., Suzuki, T., Francheschetti, S., Riggio, C., Avanzini, G., Rabinowicz, A., Bohlega, S., Bailey, J., Alonso, M.E., Rasmussen, A., Thomson, A.E., Ochoa, A., Prado, A.J., Medina, M.T., and Yamakawa, K. (2002). Genotype-phenotype correlations for EPM2A mutations in Lafora's progressive myoclonus epilepsy: exon 1 mutations associate with an early-onset cognitive deficit subphenotype. *Hum. Mol. Genet.* 11:1263–1271.
- Garcia-Mata, R., Bebok, Z., Sorscher, E.J., and Sztul, E.S. (1999). Characterization and dynamics of aggresome formation by a cytosolic GFP-chimera. *J. Cell Biol.* 146:1239–1254.
- Gasser, T. (2001). Genetics of Parkinson's disease. *J. Neurol.* 248:833–840.
- Glover, J.R., and Lindquist, S. (1998). Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. *Cell* 94:73–82.
- Goldfarb, L.G., Park, K.Y., Cervenakova, L., Gorokhova, S., Lee, H.S., Vasconcelos, O., Nagle, J.W., Semino-Mora, C., Sivakumar, K., and Dalakas, M.C. (1998). Missense mutations in desmin associated with familial cardiac and skeletal myopathy. *Nat. Genet.* 19:402–403.
- Goldfarb, L.G., Vicart, P., Goebel, H.H., and Dalakas, M.C. (2004). Desmin myopathy. *Brain* 127:723–734.
- Goldman, R.D., Chou, Y.H., Prahlad, V., and Yoon, M. (1999). Intermediate filaments: dynamic processes regulating their assembly, motility, and interactions with other cytoskeletal systems. *FASEB J.* 13(Suppl 2):S261–S265.
- Goldstein, L.S., and Yang, Z. (2000). Microtubule-based transport systems in neurons: the roles of kinesins and dyneins. *Annu. Rev. Neurosci.* 23:39–71.
- Gong, X., Li, E., Klier, G., Huang, Q., Wu, Y., Lei, H., Kumar, N.M., Horwitz, J., and Gilula, N.B. (1997). Disruption of alpha3 connexin gene leads to proteolysis and cataractogenesis in mice. *Cell* 91:833–843.
- Goodenough, D.A. (1992). The crystalline lens. A system networked by gap junctional intercellular communication. *Semin. Cell Biol.* 3:49–58.
- Goodenough, D.A., Goliger, J.A., and Paul, D.L. (1996). Connexins, connexons, and intercellular communication. *Annu. Rev. Biochem.* 65:475–502.
- Gorrie, G.H., Vallis, Y., Stephenson, A., Whitfield, J., Browning, B., Smart, T.G., and Moss, S.J. (1997). Assembly of GABAA receptors composed of alpha1 and beta2 subunits in both cultured neurons and fibroblasts. *J. Neurosci.* 17:6587–6596.
- Grant, P., Sharma, P., and Pant, H.C. (2001). Cyclin-dependent protein kinase 5 (Cdk5) and the regulation of neurofilament metabolism. *Eur. J. Biochem.* 268:1534–1546.
- Gray, D.A., Tsirigotis, M., and Woulfe, J. (2003). Ubiquitin, proteasomes, and the aging brain. *Sci. Aging Knowl. Environ.* 2003:RE6.
- Groll, M., Bajorek, M., Kohler, A., Moroder, L., Rubin, D.M., Huber, R., Glickman, M.H., and Finley, D. (2000). A gated channel into the proteasome core particle [see comment]. *Nat. Struct. Biol.* 7:1062–1067.
- Gu, W.J., Corti, O., Araujo, F., Hampe, C., Jacquier, S., Lucking, C.B., Abbas, N., Duyckaerts, C., Rooney, T., Pradier, L., Ruberg, M., and Brice, A. (2003). The C289G and C418R missense mutations cause rapid sequestration of human Parkin into insoluble aggregates. *Neurobiol. Dis.* 14:357–364.
- Gurney, M.E. (1994). Transgenic-mouse model of amyotrophic lateral sclerosis[comment]. *N. Engl. J. Med.* 331:1721–1722.
- Gurney, M.E. (2000). What transgenic mice tell us about neurodegenerative disease. *Bioessays* 22:297–304.
- Gutekunst, C.A., Li, S.H., Yi, H., Ferrante, R.J., Li, X.J., and Hersch, S.M. (1998). The cellular and subcellular localization of huntingtin-associated protein 1 (HAP1): comparison with huntingtin in rat and human. *J. Neurosci.* 18:7674–7686.
- Gutekunst, C.A., Li, S.H., Yi, H., Mulroy, J.S., Kuemmerle, S., Jones, R., Rye, D., Ferrante, R.J., Hersch, S.M., and Li, X.J. (1999). Nuclear and neuropil aggregates in Huntington's disease: relationship to neuropathology. *J. Neurosci.* 19:2522–2534.
- Hansson, O., Petersen, A., Leist, M., Nicotera, P., Castilho, R.F., and Brundin, P. (1999). Transgenic mice expressing a Huntington's disease mutation are resistant to quinolinic acid-induced striatal excitotoxicity. *Proc. Natl. Acad. Sci. USA* 96:8727–8732.
- Harada, M., Sakisaka, S., Terada, K., Kimura, R., Kawaguchi, T., Koga, H., Kim, M., Taniguchi, E., Hanada, S., Suganuma, T., Furuta, K., Sugiyama, T., and Sata, M. (2001). A mutation of the Wilson disease protein, ATP7B, is degraded in the proteasomes and forms protein aggregates [comment]. *Gastroenterology* 120:967–974.
- Harper, P.S. (1991). *Huntington's disease*. London: W.B. Saunders.
- Harris, W.A., and Holt, C.E. (1999). Neurobiology. Slit, the midline repellent. *Nature* 398:462–463.
- Hatakeyama, S., and Nakayama, K.I. (2003). U-box proteins as a new family of ubiquitin ligases. *Biochem. Biophys. Res. Commun.* 302:635–645.

- Hattori, N., Shimura, H., Kubo, S., Wang, M., Shimizu, N., Tanaka, K., and Mizuno, Y. (2000). Importance of familial Parkinson's disease and parkinsonism to the understanding of nigral degeneration in sporadic Parkinson's disease. *J. Neural Trans. Suppl.*:101–116.
- Heath, C.M., Windsor, M., and Wileman, T. (2001). Aggresomes resemble sites specialized for virus assembly. *J. Cell Biol.* 153:449–455.
- Hegde, A.N., and DiAntonio, A. (2002). Ubiquitin and the synapse. *Nat. Rev. Neurosci.* 3:854–861.
- Hegde, A.N., Inokuchi, K., Pei, W., Casadio, A., Ghirardi, M., Chain, D.G., Martin, K.C., Kandel, E.R., and Schwartz, J.H. (1997). Ubiquitin C-terminal hydrolase is an immediate-early gene essential for long-term facilitation in *Aplysia*. *Cell* 89:115–126.
- Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. *Annu. Rev. Biochem.* 67:425–479.
- Hershko, A., Ciechanover, A., and Varshavsky, A. (2000). Basic Medical Research Award. The ubiquitin system. *Nat. Med.* 6:1073–1081.
- Hicke, L., and Dunn, R. (2003). Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu. Rev. Cell Dev. Biol.* 19:141–172.
- Hishikawa, N., Niwa, J., Doyu, M., Ito, T., Ishigaki, S., Hashizume, Y., and Sobue, G. (2003). Dofrin localizes to the ubiquitinated inclusions in Parkinson's disease, dementia with Lewy bodies, multiple system atrophy, and amyotrophic lateral sclerosis. *Am. J. Pathol.* 163:609–619.
- Hoffman, E.K., Wilcox, H.M., Scott, R.W., and Siman, R. (1996). Proteasome inhibition enhances the stability of mouse Cu/Zn superoxide dismutase with mutations linked to familial amyotrophic lateral sclerosis. *J. Neurol. Sci.* 139:15–20.
- Holcombe, H., Mellman, I., Janeway, C.A., Jr., Bottomly, K., and Dittell, B.N. (2002). The immunosuppressive agent 15-deoxyspergualin functions by inhibiting cell cycle progression and cytokine production following naive T cell activation. *J. Immunol.* 169:4982–4989.
- Horwich, A. (2002). Protein aggregation in disease: a role for folding intermediates forming specific multimeric interactions. *J. Clin. Invest.* 110:1221–1232.
- Howland, D.S., Liu, J., She, Y., Goad, B., Maragakis, N.J., Kim, B., Erickson, J., Kulik, J., DeVito, L., Psaltis, G., DeGennaro, L.J., Cleveland, D.W., and Rothstein, J.D. (2002). Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). *Proc. Natl. Acad. Sci. USA* 99:1604–1609.
- Huang, Y., Baker, R.T., and Fischer-Vize, J.A. (1995). Control of cell fate by a deubiquitinating enzyme encoded by the fat facets gene. *Science* 270:1828–1831.
- Huang, Y.Y., Nguyen, P.V., Abel, T., and Kandel, E.R. (1996). Long-lasting forms of synaptic potentiation in the mammalian hippocampus. *Learn. Mem.* 3:74–85.
- Hubbert, C., Guardiola, A., Shao, R., Kawaguchi, Y., Ito, A., Nixon, A., Yoshida, M., Wang, X.F., and Yao, T.P. (2002). HDAC6 is a microtubule-associated deacetylase [see comment]. *Nature* 417:455–458.
- Huh, K.H., and Wenthold, R.J. (1999). Turnover analysis of glutamate receptors identifies a rapidly degraded pool of the N-methyl-D-aspartate receptor subunit, NR1, in cultured cerebellar granule cells. *J. Biol. Chem.* 274:151–157.
- Hyun, D.H., Lee, M., Halliwell, B., and Jenner, P. (2003). Proteasomal inhibition causes the formation of protein aggregates containing a wide range of proteins, including nitrated proteins. *J. Neurochem.* 86:363–373.
- Illing, M.E., Rajan, R.S., Bence, N.F., and Kopito, R.R. (2002). A rhodopsin mutant linked to autosomal dominant retinitis pigmentosa is prone to aggregate and interacts with the ubiquitin proteasome system. *J. Biol. Chem.* 277:34150–34160.
- Imai, Y., Soda, M., and Takahashi, R. (2000). Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin–protein ligase activity. *J. Biol. Chem.* 275:35661–35664.
- Imai, Y., Soda, M., Hatakeyama, S., Akagi, T., Hashikawa, T., Nakayama, K.I., and Takahashi, R. (2002). CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. *Mol. Cell* 10:55–67.
- Ingano, L.A., Lentini, K.M., Kovacs, I., Tanzi, R.E., and Kovacs, D.M. (2000). Cytoplasmic presenilin aggregates in proteasome inhibitor-treated cells. *Ann. N Y Acad. Sci.* 920:259–260.
- Ingham, R.J., Gish, G., and Pawson, T. (2004). The Nedd4 family of E3 ubiquitin ligases: functional diversity within a common modular architecture. *Oncogene* 23:1972–1984.
- Ito, H., Okamoto, K., Nakayama, H., Isobe, T., and Kato, K. (1997). Phosphorylation of alphaB-crystallin in response to various types of stress. *J. Biol. Chem.* 272:29934–29941.
- Ito, H., Kamei, K., Iwamoto, I., Inaguma, Y., Garcia-Mata, R., Sztul, E., and Kato, K. (2002). Inhibition of proteasomes induces accumulation, phosphorylation, and recruitment of HSP27 and alphaB-crystallin to aggresomes. *J. Biochem.* 131:593–603.

- Ito, H., Kamei, K., Iwamoto, I., Inaguma, Y., Tsuzuki, M., Kishikawa, M., Shimada, A., Hosokawa, M., and Kato, K. (2003). Hsp27 suppresses the formation of inclusion bodies induced by expression of R120G alpha B-crystallin, a cause of desmin-related myopathy. *Cell. Mol. Life Sci.* 60:1217–1223.
- Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., Maxwell, P.H., Pugh, C.W., and Ratcliffe, P.J. (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation [see comment]. *Science* 292:468–472.
- Jessenberger, V., and Jentsch, S. (2002). Deadly encounter: ubiquitin meets apoptosis. *Nat. Rev. Mol. Cell Biol.* 3:112–121.
- Jin, T., Gu, Y., Zanusso, G., Sy, M., Kumar, A., Cohen, M., Gambetti, P., and Singh, N. (2000). The chaperone protein BiP binds to a mutant prion protein and mediates its degradation by the proteasome. *J. Biol. Chem.* 275: 38699–38704.
- Joazeiro, C.A., and Weissman, A.M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. *Cell* 102: 549–552.
- Johnson, E.S., Bartel, B., Seufert, W., and Varshavsky, A. (1992). Ubiquitin as a degradation signal. *EMBO J.* 11: 497–505.
- Johnston, J.A., and Madura, K.I. (2004). Rings, chains and ladders: Ubiquitin goes to work in the neuron. *Prog. Neurobiol.* 73:227–257.
- Johnston, J.A., Johnson, E.S., Waller, P.R., and Varshavsky, A. (1995). Methotrexate inhibits proteolysis of dihydrofolate reductase by the N-end rule pathway. *J. Biol. Chem.* 270:8172–8178.
- Johnston, J.A., Ward, C.L., and Kopito, R.R. (1998). Aggresomes: a cellular response to misfolded proteins. *J. Cell Biol.* 143:1883–1898.
- Johnston, J.A., Dalton, M.J., Gurney, M.E., and Kopito, R.R. (2000). Formation of high molecular weight complexes of mutant Cu, Zn-superoxide dismutase in a mouse model for familial amyotrophic lateral sclerosis. *Proc. Natl. Acad. Sci. USA* 97:12571–12576.
- Johnston, J.A., Illing, M.E., and Kopito, R.R. (2002). Cytoplasmic dynein/dynactin mediates the assembly of aggresomes. *Cell Motil. Cytoskel.* 53:26–38.
- Jones, D., Crowe, E., Stevens, T.A., and Candido, E.P. (2002). Functional and phylogenetic analysis of the ubiquitylation system in *Caenorhabditis elegans*: ubiquitin-conjugating enzymes, ubiquitin-activating enzymes, and ubiquitin-like proteins. *Genome Biol.* 3:RESEARCH0002.
- Kabore, A.F., Wang, W.J., Russo, S.J., and Beers, M.F. (2001). Biosynthesis of surfactant protein C: characterization of aggresome formation and cell viability in response to misfolded protein stress. *J. Cell Sci.* 114:293–302.
- Kahns, S., Kalai, M., Jakobsen, L.D., Clark, B.F., Vandenabeele, P., and Jensen, P.H. (2003). Caspase-1 and caspase-8 cleave and inactivate cellular parkin. *J. Biol. Chem.* 278:23376–23380.
- Kato, K., Ito, H., Kamei, K., Inaguma, Y., Iwamoto, I., and Saga, S. (1998). Phosphorylation of alphaB-crystallin in mitotic cells and identification of enzymatic activities responsible for phosphorylation. *J. Biol. Chem.* 273: 28346–28354.
- Kato, K., Ito, H., Kamei, K., and Iwamoto, I. (1999). Selective stimulation of Hsp27 and alphaB-crystallin but not Hsp70 expression by p38 MAP kinase activation. *Cell Stress Chaperones* 4:94–101.
- Kaufman, R.J. (1999). Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev.* 13:1211–1233.
- Kawaguchi, Y., Kovacs, J.J., McLaurin, A., Vance, J.M., Ito, A., and Yao, T.P. (2003). The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. *Cell* 115:727–738.
- Kawajiri, A., Yasui, Y., Goto, H., Tatsuka, M., Takahashi, M., Nagata, K., and Inagaki, M. (2003). Functional significance of the specific sites phosphorylated in desmin at cleavage furrow: Aurora-B may phosphorylate and regulate type III intermediate filaments during cytokinesis coordinately with Rho-kinase. *Mol. Biol. Cell* 14:1489–1500.
- Kesavapany, S., Li, B.S., Amin, N., Zheng, Y.L., Grant, P., and Pant, H.C. (2004). Neuronal cyclin-dependent kinase 5: role in nervous system function and its specific inhibition by the Cdk5 inhibitory peptide. *Biochim. Biophys. Acta* 1697:143–153.
- Kirby, J., Menzies, F.M., Cookson, M.R., Bushby, K., and Shaw, P.J. (2002). Differential gene expression in a cell culture model of SOD1-related familial motor neurone disease. *Hum. Mol. Genet.* 11:2061–2075.
- Kisselev, A.F., Akopian, T.N., Castillo, V., and Goldberg, A.L. (1999). Proteasome active sites allosterically regulate each other, suggesting a cyclical bite-chew mechanism for protein breakdown. *Mol. Cell* 4:395–402.

- Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism [see comment]. *Nature* 392:605–608.
- Klemenz, R., Frohli, E., Steiger, R.H., Schafer, R., and Aoyama, A. (1991). Alpha B-crystallin is a small heat shock protein. *Proc. Natl. Acad. Sci. USA* 88:3652–3656.
- Kloetzel, P.M. (2001). Antigen processing by the proteasome. *Nat. Rev. Mol. Cell Biol.* 2:179–187.
- Koepp, D.M., Harper, J.W., and Elledge, S.J. (1999). How the cyclin became a cyclin: regulated proteolysis in the cell cycle. *Cell* 97:431–434.
- Konietzko, U., Kauselmann, G., Scafidi, J., Staubli, U., Mikkers, H., Berns, A., Schweizer, M., Waltereit, R., and Kuhl, D. (1999). Pim kinase expression is induced by LTP stimulation and required for the consolidation of enduring LTP. *EMBO J.* 18:3359–3369.
- Korlipara, L.V., and Schapira, A.H. (2002). Parkinson's disease. *Int. Rev. Neurobiol.* 53:283–314.
- Kostova, Z., and Wolf, D.H. (2003). For whom the bell tolls: protein quality control of the endoplasmic reticulum and the ubiquitin–proteasome connection. *EMBO J.* 22:2309–2317.
- Kregel, K.C. (2002). Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *J. Appl. Physiol.* 92:2177–2186.
- Kruger, E., Kloetzel, P.M., and Enenkel, C. (2001). 20S proteasome biogenesis. *Biochimie* 83:289–293.
- Kuusisto, E., Salminen, A., and Alafuzoff, I. (2001a). Ubiquitin-binding protein p62 is present in neuronal and glial inclusions in human tauopathies and synucleinopathies. *Neuroreport* 12:2085–2090.
- Kuusisto, E., Suuronen, T., and Salminen, A. (2001b). Ubiquitin-binding protein p62 expression is induced during apoptosis and proteasomal inhibition in neuronal cells. *Biochem. Biophys. Res. Commun.* 280:223–228.
- Kuusisto, E., Parkkinen, L., and Alafuzoff, I. (2003). Morphogenesis of Lewy bodies: dissimilar incorporation of alpha-synuclein, ubiquitin, and p62. *J. Neuropathol. Exp. Neurol.* 62:1241–1253.
- Kuzuhara, S., Mori, H., Izumiya, N., Yoshimura, M., and Ihara, Y. (1988). Lewy bodies are ubiquitinated. A light and electron microscopic immunocytochemical study. *Acta Neuropathol.* 75:345–353.
- Kwak, S., Masaki, T., Ishiura, S., and Sugita, H. (1991). Multicatalytic proteinase is present in Lewy bodies and neurofibrillary tangles in diffuse Lewy body disease brains. *Neurosci. Lett.* 128:21–24.
- Lam, Y.A., Pickart, C.M., Alban, A., Landon, M., Jamieson, C., Ramage, R., Mayer, R.J., and Layfield, R. (2000). Inhibition of the ubiquitin-proteasome system in Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* 97:9902–9906.
- Lam, Y.A., Lawson, T.G., Velayutham, M., Zweier, J.L., and Pickart, C.M. (2002). A proteasomal ATPase subunit recognizes the polyubiquitin degradation signal. *Nature* 416:763–767.
- Lee, C., Schwartz, M.P., Prakash, S., Iwakura, M., and Matouschek, A. (2001). ATP-dependent proteases degrade their substrates by processively unraveling them from the degradation signal. *Mol. Cell* 7:627–637.
- Lee, H.J., and Lee, S.J. (2002). Characterization of cytoplasmic alpha-synuclein aggregates. Fibril formation is tightly linked to the inclusion-forming process in cells. *J. Biol. Chem.* 277:48976–48983.
- Lee, H.J., Shin, S.Y., Choi, C., Lee, Y.H., and Lee, S.J. (2002). Formation and removal of alpha-synuclein aggregates in cells exposed to mitochondrial inhibitors. *J. Biol. Chem.* 277:5411–5417.
- Leggett, D.S., Hanna, J., Borodovsky, A., Crosas, B., Schmidt, M., Baker, R.T., Walz, T., Ploegh, H., and Finley, D. (2002). Multiple associated proteins regulate proteasome structure and function. *Mol. Cell* 10:495–507.
- Lelouard, H., Gatti, E., Cappello, F., Gresser, O., Camosseto, V., and Pierre, P. (2002). Transient aggregation of ubiquitinated proteins during dendritic cell maturation. *Nature* 417:177–182.
- Lelouard, H., Ferrand, V., Marguet, D., Bania, J., Camosseto, V., David, A., Gatti, E., and Pierre, P. (2004). Dendritic cell aggresome-like induced structures are dedicated areas for ubiquitination and storage of newly synthesized defective proteins. *J. Cell Biol.* 164:667–675.
- Leterrier, J.F., Kas, J., Hartwig, J., Vegners, R., and Janmey, P.A. (1996). Mechanical effects of neurofilament cross-bridges. Modulation by phosphorylation, lipids, and interactions with F-actin. *J. Biol. Chem.* 271:15687–15694.
- Levitskaya, J., Sharipo, A., Leonchiks, A., Ciechanover, A., and Masucci, M.G. (1997). Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1. *Proc. Natl. Acad. Sci. USA* 94:12616–12621.
- Li, J., Uversky, V.N., and Fink, A.L. (2001). Effect of familial Parkinson's disease point mutations A30P and A53T on the structural properties, aggregation, and fibrillation of human alpha-synuclein. *Biochemistry* 40:11604–11613.
- Li, R., Zheng, Y., and Drubin, D.G. (1995). Regulation of cortical actin cytoskeleton assembly during polarized cell growth in budding yeast. *J. Cell Biol.* 128:599–615.

- Li, S.H., Gutekunst, C.A., Hersch, S.M., and Li, X.J. (1998). Interaction of huntingtin-associated protein with dynactin P150Glued. *J. Neurosci.* 18:1261–12169.
- Linden, D.J. (1999). The return of the spike: postsynaptic action potentials and the induction of LTP and LTD. *Neuron* 22:661–666.
- Linden, D.J., and Connor, J.A. (1995). Long-term synaptic depression. *Annu. Rev. Neurosci.* 18:319–357.
- Lindquist, S., and Kim, G. (1996). Heat-shock protein 104 expression is sufficient for thermotolerance in yeast. *Proc. Natl. Acad. Sci. USA* 93:5301–5306.
- Lindsten, K., de Vrij, F.M., Verhoef, L.G., Fischer, D.F., van Leeuwen, F.W., Hol, E.M., Masucci, M.G., and Dantuma, N.P. (2002). Mutant ubiquitin found in neurodegenerative disorders is a ubiquitin fusion degradation substrate that blocks proteasomal degradation. *J. Cell Biol.* 157:417–427.
- Lindsten, K., Menendez-Benito, V., Masucci, M.G., and Dantuma, N.P. (2003). A transgenic mouse model of the ubiquitin/proteasome system. *Nat. Biotechnol.* 21:897–902.
- Lorick, K.L., Jensen, J.P., Fang, S., Ong, A.M., Hatakeyama, S., and Weissman, A.M. (1999). RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc. Natl. Acad. Sci. USA* 96:11364–11369.
- Luby-Phelps, K. (2000). Cytoarchitecture and physical properties of cytoplasm: volume, viscosity, diffusion, intracellular surface area. *Int. Rev. Cytol.* 192:189–221.
- Lunkes, A., and Mandel, J.L. (1998). A cellular model that recapitulates major pathogenic steps of Huntington's disease. *Hum. Mol. Genet.* 7:1355–1361.
- Lupas, A., Koster, A.J., and Baumeister, W. (1993). Structural features of 26S and 20S proteasomes. *Enzyme Protein* 47:252–273.
- Lupski, J.R., and Garcia, C.A. (2001). Charcot-marie-tooth peripheral neuropathies and related disorders. In: Scrivner, C., Beaudet, A., Valle, D., Sly, W., Childs, B., Kinzler, K., and Vogelstein, B. (eds.), *The metabolic and molecular bases of inherited disease*. New York: McGraw-Hill, pp. 5759–5788.
- Ma, J., and Lindquist, S. (2001). Wild-type PrP and a mutant associated with prion disease are subject to retrograde transport and proteasome degradation. *Proc. Natl. Acad. Sci. USA* 98:14955–14960.
- Ma, J., and Lindquist, S. (2002). Conversion of PrP to a self-perpetuating PrP^{Sc}-like conformation in the cytosol [see comment]. *Science* 298:1785–1788.
- Ma, J., Wollmann, R., and Lindquist, S. (2002). Neurotoxicity and neurodegeneration when PrP accumulates in the cytosol [see comment]. *Science* 298:1781–1785.
- Magin, T. M., Schroder, R., Leitgeb, S., Wanninger, F., Zatloukal, K., Grund, C., and Melton, D. W. (1998). Lessons from keratin 18 knockout mice: formation of novel keratin filaments, secondary loss of keratin 7 and accumulation of liver-specific keratin 8-positive aggregates. *J. Cell Biol.* 140:1441–1451.
- Malinow, R., and Malenka, R.C. (2002). AMPA receptor trafficking and synaptic plasticity. *Annu. Rev. Neurosci.* 25:103–126.
- Malone, C.J., Misner, L., Le Bot, N., Tsai, M.C., Campbell, J.M., Ahringer, J., and White, J.G. (2003). The *C. elegans* hook protein, ZYG-12, mediates the essential attachment between the centrosome and nucleus. *Cell* 115:825–836.
- Manganas, L.N., Akhtar, S., Antonucci, D.E., Campomanes, C.R., Dolly, J.O., and Trimmer, J.S. (2001). Episodic ataxia type-1 mutations in the Kv1.1 potassium channel display distinct folding and intracellular trafficking properties. *J. Biol. Chem.* 276:49427–49434.
- Maniatis, T. (1999). A ubiquitin ligase complex essential for the NF-kappaB, Wnt/Wingless, and Hedgehog signaling pathways. *Genes Dev.* 13:505–510.
- Maro, B. and Bornens, M. (1980). The centriole–nucleus association: effects of cytochalasin B and nocodazole. *Biol. Cell* 39:287–290.
- Martin-Aparicio, E., Yamamoto, A., Hernandez, F., Hen, R., Avila, J., and Lucas, J.J. (2001). Proteasomal-dependent aggregate reversal and absence of cell death in a conditional mouse model of Huntington's disease. *J. Neurosci.* 21:8772–8781.
- Martin-Aparicio, E., Avila, J., and Lucas, J.J. (2002). Nuclear localization of N-terminal mutant huntingtin is cell cycle dependent. *Eur. J. Neurosci.* 16:355–359.
- Matsuoka, Y., Nishizawa, K., Yano, T., Shibata, M., Ando, S., Takahashi, T., and Inagaki, M. (1992). Two different protein kinases act on a different time schedule as glial filament kinases during mitosis. *EMBO J.* 11:2895–2902.
- Matsuyama, A., Shimazu, T., Sumida, Y., Saito, A., Yoshimatsu, Y., Seigneurin-Berny, D., Osada, H., Komatsu, Y., Nishino, N., Khochbin, S., Horinouchi, S., and Yoshida, M. (2002). In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation. *EMBO J.* 21:6820–6831.
- McCabe, B.D., Hom, S., Aberle, H., Fetter, R.D., Marques, G., Haerry, T.E., Wan, H., O'Connor, M.B., Goodman, C.S., and Haghighi, A.P. (2004). Highwire regulates presynaptic BMP signalling essential for synaptic growth. *Neuron* 41:891–905.

- McNaught, K.S., Bjorklund, L.M., Belizaire, R., Isacson, O., Jenner, P., and Olanow, C.W. (2002a). Proteasome inhibition causes nigral degeneration with inclusion bodies in rats. *Neuroreport* 13:1437–1441.
- McNaught, K.S., Mytilineou, C., Jnabaptiste, R., Yabut, J., Shashidharan, P., Jennert, P., and Olanow, C.W. (2002b). Impairment of the ubiquitin–proteasome system causes dopaminergic cell death and inclusion body formation in ventral mesencephalic cultures. *J. Neurochem.* 81:301–306.
- McNaught, K.S., Shashidharan, P., Perl, D.P., Jenner, P., and Olanow, C.W. (2002c). Aggresome-related biogenesis of Lewy bodies. *Eur. J. Neurosci.* 16:2136–2148.
- McPhaul, L.W., Wang, J., Hol, E.M., Sonnemans, M.A., Riley, N., Nguyen, V., Yuan, Q.X., Lue, Y.H., Van Leeuwen, F.W., and French, S.W. (2002). Molecular misreading of the ubiquitin B gene and hepatic mallory body formation. *Gastroenterology* 122:1878–1885.
- Meriin, A.B., Zhang, X., He, X., Newnam, G.P., Chernoff, Y.O., and Sherman, M.Y. (2002). Huntington toxicity in yeast model depends on polyglutamine aggregation mediated by a prion-like protein Rnq1 [erratum appears in *J. Cell Biol.* 2002 Aug 5;158(3):591]. *J. Cell Biol.* 157:997–1004.
- Michalek, M.T., Grant, E.P., Gramm, C., Goldberg, A.L., and Rock, K.L. (1993). A role for the ubiquitin-dependent proteolytic pathway in MHC class I-restricted antigen presentation. *Nature* 363:552–554.
- Milam, A.H., Li, Z.Y., and Fariss, R.N. (1998). Histopathology of the human retina in retinitis pigmentosa. *Prog. Retinal Eye Res.* 17:175–205.
- Mishra, R.S., Bose, S., Gu, Y., Li, R., and Singh, N. (2003). Aggresome formation by mutant prion proteins: the unfolding role of proteasomes in familial prion disorders. *J. Alzheimer's Dis.* 5:15–23.
- Mitchison, T.J., and Kirschner, M.W. (1986). Isolation of mammalian centrosomes. *Methods Enzymol.* 134:261–268.
- Mizushima, T., Hirao, T., Yoshida, Y., Lee, S.J., Chiba, T., Iwai, K., Yamaguchi, Y., Kato, K., Tsukihara, T., and Tanaka, K. (2004). Structural basis of sugar-recognizing ubiquitin ligase. *Nat. Struct. Mol. Biol.* 11:365–370.
- Monia, B.P., Ecker, D.A., and Crooke, S.T. (1990). New perspectives on the structure and function of ubiquitin. *BioTechnology* 8:209–215.
- Moon, A., and Drubin, D.G. (1995). The ADF/cofilin proteins: stimulus-responsive modulators of actin dynamics. *Mol. Biol. Cell* 6:1423–1431.
- Muchowski, P.J., Ning, K., D'Souza-Schorey, C., and Fields, S. (2002). Requirement of an intact microtubule cytoskeleton for aggregation and inclusion body formation by a mutant huntingtin fragment. *Proc. Natl. Acad. Sci. USA* 99:727–732.
- Muneoka, K.T., and Takigawa, M. (2003). 5-Hydroxytryptamine7 (5-HT7) receptor immunoreactivity-positive “stigmoid body”-like structure in developing rat brains. *Int. J. Dev. Neurosci.* 21:133–143.
- Murakami, Y., Tanahashi, N., Tanaka, K., Omura, S., and Hayashi, S. (1996). Proteasome pathway operates for the degradation of ornithine decarboxylase in intact cells. *Biochem. J.* 317:77–80.
- Murata, S., Minami, Y., Minami, M., Chiba, T. and Tanaka, K. (2001). CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. *EMBO Rep.* 2:1133–1138.
- Muresan, V. (2000). One axon, many kinesins: What's the logic? *J. Neurocytol.* 29:799–818.
- Muresan, V., Abramson, T., Lyass, A., Winter, D., Porro, E., Hong, F., Chamberlin, N.L., and Schnapp, B.J. (1998). KIF3C and KIF3A form a novel neuronal heteromeric kinesin that associates with membrane vesicles. *Mol. Biol. Cell* 9:637–652.
- Murphey, R.K., and Godenschwege, T.A. (2002). New roles for ubiquitin in the assembly and function of neuronal circuits. *Neuron* 36:5–8.
- Musil, L.S., Le, A.C., VanSlyke, J.K., and Roberts, L.M. (2000). Regulation of connexin degradation as a mechanism to increase gap junction assembly and function. *J. Biol. Chem.* 275:25207–25215.
- Narayanan, V., and Scarlata, S. (2001). Membrane binding and self-association of alpha-synucleins. *Biochemistry* 40:9927–9934.
- Navon, A., and Goldberg, A.L. (2001). Proteins are unfolded on the surface of the ATPase ring before transport into the proteasome. *Mol. Cell* 8:1339–1350.
- Okada, T., Ernst, O.P., Palczewski, K., and Hofmann, K.P. (2001). Activation of rhodopsin: new insights from structural and biochemical studies. *Trends Biochem. Sci.* 26:318–324.
- Olsen, M.K., Roberds, S.L., Ellerbrock, B.R., Fleck, T.J., McKinley, D.K., and Gurney, M.E. (2001). Disease mechanisms revealed by transcription profiling in SOD1-G93A transgenic mouse spinal cord. *Ann. Neurol.* 50:730–740.
- Ostrerova-Golts, N., Petrucelli, L., Hardy, J., Lee, J.M., Farer, M., and Wolozin, B. (2000). The A53T alpha-synuclein mutation increases iron-dependent aggregation and toxicity. *J. Neurosci.* 20:6048–6054.
- Pak, D.T., and Sheng, M. (2003). Targeted protein degradation and synapse remodeling by an inducible protein kinase. *Science* 302:1368–1373.

- Pak, D.T., Yang, S., Rudolph-Correia, S., Kim, E., and Sheng, M. (2001). Regulation of dendritic spine morphology by SPAR, a PSD-95-associated RapGAP [comment]. *Neuron* 31:289–303.
- Pal, J.D., Berthoud, V.M., Beyer, E.C., Mackay, D., Shiels, A., and Ebihara, L. (1999). Molecular mechanism underlying a Cx50-linked congenital cataract [erratum appears in *Am. J. Physiol.* 1999 Dec;277(6 Pt 1):section C]. *Am. J. Physiol.* 276:C1443–C1446.
- Pareek, S., Notterpek, L., Snipes, G.J., Naef, R., Sossin, W., Laliberte, J., Iacampo, S., Suter, U., Shooter, E.M., and Murphy, R.A. (1997). Neurons promote the translocation of peripheral myelin protein 22 into myelin. *J. Neurosci.* 17:7754–7762.
- Patil, C., and Walter, P. (2001). Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. *Curr. Opin. Cell Biol.* 13:349–355.
- Paxinou, E., Chen, Q., Weisse, M., Giasson, B.I., Norris, E.H., Rueter, S.M., Trojanowski, J.Q., Lee, V.M., and Ischiropoulos, H. (2001). Induction of alpha-synuclein aggregation by intracellular nitrate insult. *J. Neurosci.* 21:8053–8061.
- Perutz, M.F., Johnson, T., Suzuki, M., and Finch, J.T. (1994). Glutamine repeats as polar zippers: their possible role in inherited neurodegenerative diseases. *Proc. Natl. Acad. Sci. USA* 21:5355–5358.
- Petaja-Repo, U.E., Hogue, M., Laperriere, A., Bhalla, S., Walker, P., and Bouvier, M. (2001). Newly synthesized human delta opioid receptors retained in the endoplasmic reticulum are retrotranslocated to the cytosol, deglycosylated, ubiquitinated, and degraded by the proteasome. *J. Biol. Chem.* 276:4416–4423.
- Piao, Y.S., Wakabayashi, K., Kakita, A., Yamada, M., Hayashi, S., Morita, T., Ikuta, F., Oyanagi, K., and Takahashi, H. (2003). Neuropathology with clinical correlations of sporadic amyotrophic lateral sclerosis: 102 autopsy cases examined between 1962 and 2000. *Brain Pathol.* 13:10–22.
- Pickart, C.M. (2000). Ubiquitin in chains. *Trends Biochem. Sci.* 25:544–548.
- Poeck, B., Fischer, S., Gunning, D., Zipursky, S.L., and Salecker, I. (2001). Glial cells mediate target layer selection of retinal axons in the developing visual system of *Drosophila*. *Neuron* 29:99–113.
- Polyakov, A.V., Shagina, I.A., Khlebnikova, O.V., and Evgrafov, O.V. (2001). Mutation in the connexin 50 gene (GJA8) in a Russian family with zonular pulverulent cataract. *Clin. Genet.* 60:476–478.
- Polymeropoulos, M.H., Lavendan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, F., Stenroos, E.S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W.G., Lazzarini, A.M., Duvoisin, R.C., Dilorio, G., Golbe, L.I., and Nussbaum, R.L. (1997). Mutation in the a-synuclein gene identified in families with Parkinson's disease. *Science* 276:2045–2047.
- Prusiner, S.B. (1998). The prion diseases. *Brain Pathol.* 8:499–513.
- Prusiner, S.B., Scott, M.R., DeArmond, S.J., and Cohen, F.E. (1998). Prion protein biology. *Cell* 93:337–348.
- Rajan, R.S., Illing, M.E., Bence, N.F., and Kopito, R.R. (2001). Specificity in intracellular protein aggregation and inclusion body formation. *Proc. Natl. Acad. Sci. USA* 98:13060–13065.
- Rideout, H.J., Larsen, K.E., Sulzer, D., and Stefanis, L. (2001). Proteasomal inhibition leads to formation of ubiquitin/alpha-synuclein-immunoreactive inclusions in PC12 cells. *J. Neurochem.* 78:899–908.
- Rieder, C.L., Faruki, S., and Khodjakov, A. (2001). The centrosome in vertebrates: more than a microtubule-organizing center. *Trends Cell Biol.* 11:413–419.
- Riley, N.E., Bardag-Gorce, F., Montgomery, R.O., Li, J., Lungu, W., Lue, Y.H., and French, S.W. (2003). Microtubules are required for cytokeratin aggresome (Mallory body) formation in hepatocytes: an in vitro study. *Exp. Mol. Pathol.* 74:173–179.
- Rock, K.L., and Goldberg, A.L. (1999). Degradation of cell proteins and the generation of MHC class I-presented peptides. *Annu. Rev. Immunol.* 17:739–779.
- Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A.L. (1994). Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* 78:761–771.
- Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., and Deng, H.X. (1993). Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis [see comment] [erratum appears in *Nature* 1993 Jul 22;364(6435):362; PMID: 8332197]. *Nature* 362:59–62.
- Rubin, D.M., van Nocker, S., Glickman, M., Coux, O., Wefes, I., Sadis, S., Fu, H., Goldberg, A., Vierstra, R., and Finley, D. (1997). ATPase and ubiquitin-binding proteins of the yeast proteasome. *Mol. Biol. Rep.* 24:17–26.
- Ryan, M.C., Shooter, E.M., and Notterpek, L. (2002). Aggresome formation in neuropathy models based on peripheral myelin protein 22 mutations. *Neurobiol. Dis.* 10:109–118.
- Sakata, E., Yamaguchi, Y., Kurimoto, E., Kikuchi, J., Yokoyama, S., Yamada, S., Kawahara, H., Yokosawa, H., Hattori, N., Mizuno, Y., Tanaka, K., and Kato, K. (2003). Parkin binds the Rpn10 subunit of 26S proteasomes through its ubiquitin-like domain. *EMBO Rep.* 4:301–306.

- Saliba, R.S., Munro, P.M., Luthert, P.J., and Cheetham, M.E. (2002). The cellular fate of mutant rhodopsin: quality control, degradation and aggresome formation. *J. Cell Sci.* 115:2907–2918.
- Sampathu, D.M., Giasson, B.I., Pawlyk, A.C., Trojanowski, J.Q., and Lee, V.M. (2003). Ubiquitination of alpha-synuclein is not required for formation of pathological inclusions in alpha-synucleinopathies. *Am. J. Pathol.* 163:91–100.
- Scherzinger, E., Sittler, A., Schweiger, K., Heiser, V., Lurz, R., Hasenbank, R., Bates, G.P., Lehrach, H., and Wanker, E.E. (1999). Self-assembly of polyglutamine-containing huntingtin fragments into amyloid-like fibrils: implications for Huntington's disease pathology. *Proc. Natl. Acad. Sci. USA* 96:4604–4609.
- Schiene-Fischer, C., and Yu, C. (2001). Receptor accessory folding helper enzymes: the functional role of peptidyl prolyl *cis/trans* isomerases. *FEBS Lett.* 495:1–6.
- Schlossmacher, M.G., Frosch, M.P., Gai, W.P., Medina, M., Sharma, N., Forno, L., Ochiishi, T., Shimura, H., Sharon, R., Hattori, N., Langston, J.W., Mizuno, Y., Hyman, B.T., Selkoe, D.J., and Kosik, K.S. (2002). Parkin localizes to the Lewy bodies of Parkinson disease and dementia with Lewy bodies. *Am. J. Pathol.* 160:1655–1667.
- Schreiber, S.L. (1991). Chemistry and biology of the immunophilins and their immunosuppressive ligands. *Science* 251: 283–287.
- Sekimata, M., Tsujimura, K., Tanaka, J., Takeuchi, Y., Inagaki, N., and Inagaki, M. (1996). Detection of protein kinase activity specifically activated at metaphase-anaphase transition. *J. Cell Biol.* 132:635–641.
- Seiple, C.A., Group, R.G., and Members, G.S.L. (2003). The comparative proteomics of ubiquitination in mouse. *Genome Res.* 13:1389–1394.
- Serrando, M., Casanovas, A., and Esquerda, J.E. (2002). Occurrence of glutamate receptor subunit 1-containing aggresome-like structures during normal development of rat spinal cord interneurons. *J. Comp. Neurol.* 442: 23–34.
- Sharon, R., Goldberg, M.S., Bar-Josef, I., Betensky, R.A., Shen, J., and Selkoe, D.J. (2001). alpha-Synuclein occurs in lipid-rich high molecular weight complexes, binds fatty acids, and shows homology to the fatty acid-binding proteins. *Proc. Natl. Acad. Sci. USA* 98:9110–9115.
- Shenoy, S.K., and Lefkowitz, R.J. (2003). Trafficking patterns of beta-arrestin and G protein-coupled receptors determined by the kinetics of beta-arrestin deubiquitination. *J. Biol. Chem.* 278:14498–14506.
- Shenoy, S.K., McDonald, P.H., Kohout, T.A., and Lefkowitz, R.J. (2001). Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. *Science* 294:1307–1313.
- Sherman, M.Y., and Goldberg, A.L. (2001). Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. *Neuron* 29:15–32.
- Shibata, N., Hirano, A., Kobayashi, M., Siddique, T., Deng, H.X., Hung, W.Y., Kato, T., and Asayama, K. (1996). Intense superoxide dismutase-1 immunoreactivity in intracytoplasmic hyaline inclusions of familial amyotrophic lateral sclerosis with posterior column involvement. *J. Neuropathol. Exp. Neurol.* 55:481–490.
- Shiels, A., Mackay, D., Ionides, A., Berry, V., Moore, A., and Bhattacharya, S. (1998). A missense mutation in the human connexin50 gene (GJA8) underlies autosomal dominant “zonular pulverulent” cataract, on chromosome 1q. *Am. J. Hum. Genet.* 62:526–532.
- Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K., and Suzuki, T. (2000). Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat. Genet.* 25:302–305.
- Shinoda, K. (1994). Sex-steroid receptor mechanism related to neuronal aromatase and the stigmoid body. *Hormones Behav.* 28:545–555.
- Siddique, T., and Hentati, A. (1995). Familial amyotrophic lateral sclerosis. *Clin. Neurosci.* 3:338–347.
- Silverstein, A.M., Galigniana, M.D., Kanelakis, K.C., Radanyi, C., Renoir, J.M., and Pratt, W.B. (1999). Different regions of the immunophilin FKBP52 determine its association with the glucocorticoid receptor, hsp90, and cytoplasmic dynein. *J. Biol. Chem.* 274:36980–36986.
- Sitte, N., Merker, K., Von Zglinicki, T., Davies, K.J., and Grune, T. (2000a). Protein oxidation and degradation during cellular senescence of human BJ fibroblasts: part II—aging of nondividing cells. *FASEB J.* 14:2503–2510.
- Sitte, N., Merker, K., Von Zglinicki, T., Grune, T., and Davies, K.J. (2000b). Protein oxidation and degradation during cellular senescence of human BJ fibroblasts: part I—effects of proliferative senescence. *FASEB J.* 14: 2495–2502.
- Skalli, O., and Goldman, R.D. (1991). Recent insights into the assembly, dynamics, and function of intermediate filament networks. *Cell Motil. Cytoskeleton* 19:67–79.
- Sloper-Mould, K.E., Jemc, J.C., Pickart, C.M., and Hicke, L. (2001). Distinct functional surface regions on ubiquitin. *J. Biol. Chem.* 276:30483–30489.
- Spillantini, M.G., Schmidt, M.L., Lee, V.M., Trojanowski, J.Q., Jakes, R., and Goedert, M. (1997). Alpha-synuclein in Lewy bodies. *Nature* 388:839–840.

- Spruck, C.H., and Strohmaier, H.M. (2002). Seek and destroy: SCF ubiquitin ligases in mammalian cell cycle control [see comment]. *Cell Cycle* 1:250–254.
- Srinivasan, A.N., Nagineni, C.N., and Bhat, S.P. (1992). alpha A-crystallin is expressed in non-ocular tissues. *J. Biol. Chem.* 267:23337–23341.
- Staropoli, J.F., McDermott, C., Martinat, C., Schulman, B., Demireva, E., and Abeliovich, A. (2003). Parkin is a component of an SCF-like ubiquitin ligase complex and protects postmitotic neurons from kainate excitotoxicity. *Neuron* 37:735–749.
- Stephen, A.G., Trausch-Azar, J.S., Ciechanover, A., and Schwartz, A.L. (1996). The ubiquitin-activating enzyme E1 is phosphorylated and localized to the nucleus in a cell cycle-dependent manner. *J. Biol. Chem.* 271:15608–15614.
- Steward, O., and Schuman, E.M. (2003). Compartmentalized synthesis and degradation of proteins in neurons. *Neuron* 40:347–359.
- Stumptner, C., Heid, H., Fuchsichler, A., Hauser, H., Mischinger, H.J., Zatloukal, K., and Denk, H. (1999). Analysis of intracytoplasmic hyaline bodies in a hepatocellular carcinoma. Demonstration of p62 as major constituent. *Am. J. Pathol.* 154:1701–1710.
- Sun, D., Leung, C.L., and Liem, R.K. (1996). Phosphorylation of the high molecular weight neurofilament protein (NF-H) by Cdk5 and p35. *J. Biol. Chem.* 271:14245–14251.
- Sung, C.H., Davenport, C.M., Hennessey, J.C., Maumenee, I.H., Jacobson, S.G., Heckenlively, J.R., Nowakowski, R., Fishman, G., Gouras, P., and Nathans, J. (1991a). Rhodopsin mutations in autosomal dominant retinitis pigmentosa. *Proc. Natl. Acad. Sci. USA* 88:6481–6485.
- Sung, C.H., Schneider, B.G., Agarwal, N., Papermaster, D.S., and Nathans, J. (1991b). Functional heterogeneity of mutant rhodopsins responsible for autosomal dominant retinitis pigmentosa. *Proc. Natl. Acad. Sci. USA* 88:8840–8844.
- Sung, C.H., Davenport, C.M., and Nathans, J. (1993). Rhodopsin mutations responsible for autosomal dominant retinitis pigmentosa. Clustering of functional classes along the polypeptide chain. *J. Biol. Chem.* 268:26645–26649.
- Tai, A.W., Chuang, J.Z., Bode, C., Wolfrum, U., and Sung, C.H. (1999). Rhodopsin's carboxy-terminal cytoplasmic tail acts as a membrane receptor for cytoplasmic dynein by binding to the dynein light chain Tctex-1. *Cell* 97:877–887.
- Tam, B.M., Moritz, O.L., Hurd, L.B., and Papermaster, D.S. (2000). Identification of an outer segment targeting signal in the COOH terminus of rhodopsin using transgenic *Xenopus laevis*. *J. Cell Biol.* 151:1369–1380.
- Tanaka, M., Kim, Y.M., Lee, G., Junn, E., Iwatsubo, T., and Mouradian, M.M. (2004). Aggresomes formed by alpha-synuclein and synphilin-1 are cytoprotective. *J. Biol. Chem.* 279:4625–4631.
- Tassin, A.M., and Bornens, M. (1999). Centrosome structure and microtubule nucleation in animal cells. *Biol. Cell* 91:343–354.
- Taylor, J.P., Tanaka, F., Robitschek, J., Sandoval, C.M., Taye, A., Markovic-Plese, S., and Fischbeck, K.H. (2003). Aggresomes protect cells by enhancing the degradation of toxic polyglutamine-containing protein. *Hum. Mol. Genet.* 12:749–757.
- Tessier-Lavigne, M., and Goodman, C.S. (1996). The molecular biology of axon guidance. *Science* 274:1123–1133.
- Thrower, J.S., Hoffman, L., Rechsteiner, M., and Pickart, C.M. (2000). Recognition of the polyubiquitin proteolytic signal. *EMBO J.* 19:94–102.
- Tobler, A.R., Liu, N., Mueller, L., and Shooter, E.M. (2002). Differential aggregation of the Trembler and Trembler J mutants of peripheral myelin protein 22. *Proc. Natl. Acad. Sci. USA* 99:483–488.
- Tofaris, G.K., Razaq, A., Ghetti, B., Lilley, K.S., and Spillantini, M.G. (2003). Ubiquitination of alpha-synuclein in Lewy bodies is a pathological event not associated with impairment of proteasome function. *J. Biol. Chem.* 278:44405–44411.
- Trimmer, P.A., Borland, M.K., Keeney, P.M., Bennett, J.P., Jr., and Parker, W.D., Jr. (2004). Parkinson's disease transgenic mitochondrial cybrids generate Lewy inclusion bodies. *J. Neurochem.* 88:800–812.
- Tsirigotis, M., Zhang, M., Chiu, R.K., Wouters, B.G., and Gray, D.A. (2001). Sensitivity of mammalian cells expressing mutant ubiquitin to protein-damaging agents. *J. Biol. Chem.* 276:46073–46078.
- Turner, G.C., and Varshavsky, A. (2000). Detecting and measuring cotranslational protein degradation in vivo. *Science* 289:2117–2220.
- Upadhy, S.C., and Hegde, A.N. (2003). A potential proteasome-interacting motif within the ubiquitin-like domain of parkin and other proteins. *Trends Biochem. Sci.* 28:280–283.
- Vadlamudi, R.K., Joung, I., Strominger, J.L., and Shin, J. (1996). p62, a phosphotyrosine-independent ligand of the SH2 domain of p56lck, belongs to a new class of ubiquitin-binding proteins. *J. Biol. Chem.* 271:20235–20237.
- Valdeira, M.L., Bernardes, C., Cruz, B., and Gerald, A. (1998). Entry of African swine fever virus into Vero cells and uncoating. *Vet. Microbiol.* 60:131–140.
- Valentine, J.S., and Hart, P.J. (2003). Misfolded CuZnSOD and amyotrophic lateral sclerosis. *Proc. Natl. Acad. Sci. USA* 100:3617–3622.

- Vallee, R.B., Williams, J.C., Varma, D., and Barnhart, L.E. (2004). Dynein: An ancient motor protein involved in multiple modes of transport. *J. Neurobiol.* 58:189–200.
- van Leeuwen, F.W., Fischer, D.F., Benne, R., and Hol, E.M. (2000). Molecular misreading. A new type of transcript mutation in gerontology. *Ann. N Y Acad. Sci.* 908:267–281.
- van Nocker, S., Sadis, S., Rubin, D.M., Glickman, M., Fu, H., Coux, O., Wefes, I., Finley, D., and Vierstra, R.D. (1996). The multiubiquitin-chain-binding protein Mcb1 is a component of the 26S proteasome in *Saccharomyces cerevisiae* and plays a nonessential, substrate-specific role in protein turnover. *Mol. Cell. Biol.* 16:6020–6028.
- VanSlyke, J.K., and Musil, L.S. (2002). Dislocation and degradation from the ER are regulated by cytosolic stress. *J. Cell Biol.* 157:381–394.
- VanSlyke, J.K., Deschenes, S.M., and Musil, L.S. (2000). Intracellular transport, assembly, and degradation of wild-type and disease-linked mutant gap junction proteins. *Mol. Biol. Cell* 11:1933–1946.
- Varshavsky, A. (1997). The N-end rule pathway of protein degradation. *Genes Cells* 2:13–28.
- Vicart, P., Caron, A., Guicheney, P., Li, Z., Prevost, M.C., Faure, A., Chateau, D., Chapon, F., Tome, F., Dupret, J.M., Paulin, D., and Fardeau, M. (1998). A missense mutation in the alphaB-crystallin chaperone gene causes a desmin-related myopathy. *Nat. Genet.* 20:92–95.
- Waelter, S., Boeddrich, A., Lurz, R., Scherzinger, E., Lueder, G., Lehrach, H., and Wanker, E.E. (2001). Accumulation of mutant huntingtin fragments in aggresome-like inclusion bodies as a result of insufficient protein degradation. *Mol. Biol. Cell* 12:1393–1407.
- Wang, E.W., Kessler, B.M., Borodovsky, A., Cravatt, B.F., Bogoy, M., Ploegh, H.L., and Glas, R. (2000). Integration of the ubiquitin-proteasome pathway with a cytosolic oligopeptidase activity. *Proc. Natl. Acad. Sci. USA* 97:9990–9995.
- Ward, C.L., Omura, S., and Kopito, R.R. (1995). Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell* 83:121–127.
- Wede, O.K., Lofgren, M., Li, Z., Paulin, D., and Arner, A. (2002). Mechanical function of intermediate filaments in arteries of different size examined using desmin deficient mice. *J. Physiol.* 540:941–949.
- Wenzel, T., and Baumeister, W. (1995). Conformational constraints in protein degradation by the 20S proteasome. *Nat. Struct. Biol.* 2:199–204.
- White, T.W., Goodenough, D.A., and Paul, D.L. (1998). Targeted ablation of connexin50 in mice results in microphthalmia and zonular pulverulent cataracts. *J. Cell Biol.* 143:815–825.
- Wiederkehr, T., Bukau, B., and Buchberger, A. (2002). Protein turnover: a CHIP programmed for proteolysis [comment]. *Curr. Biol.* 12:R26–R28.
- Wigley, W.C., Fabunmi, R.P., Lee, M.G., Marino, C.R., Muallem, S., DeMartino, G.N., and Thomas, P.J. (1999). Dynamic association of proteasomal machinery with the centrosome. *J. Cell Biol.* 145:481–490.
- Wolfrum, U., and Schmitt, A. (2000). Rhodopsin transport in the membrane of the connecting cilium of mammalian photoreceptor cells. *Cell Motil. Cytoskeleton* 46:95–107.
- Xu, J., Kao, S.Y., Lee, F.J., Song, W., Jin, L.W., and Yankner, B.A. (2002). Dopamine-dependent neurotoxicity of alpha-synuclein: a mechanism for selective neurodegeneration in Parkinson disease [see comment]. *Nat. Med.* 8:600–606.
- Yamamoto, A., Lucas, J.J., and Hen, R. (2000). Reversal of neuropathology and motor dysfunction in a conditional model of Huntington's disease [see comment]. *Cell* 101:57–66.
- Yedidia, Y., Horonchik, L., Tzaban, S., Yanai, A., and Taraboulos, A. (2001). Proteasomes and ubiquitin are involved in the turnover of the wild-type prion protein. *EMBO J.* 20:5383–5391.
- Yin, Y., Manoury, B., and Fahraeus, R. (2003). Self-inhibition of synthesis and antigen presentation by Epstein-Barr virus-encoded EBNA1 [see comment]. *Science* 301:1371–1374.
- Yoshida, Y., Chiba, T., Tokunaga, F., Kawasaki, H., Iwai, K., Suzuki, T., Ito, Y., Matsuoka, K., Yoshida, M., Tanaka, K., and Tai, T. (2002). E3 ubiquitin ligase that recognizes sugar chains. *Nature* 418:438–442.
- Zanusso, G., Petersen, R.B., Jin, T., Jing, Y., Kanoush, R., Ferrari, S., Gambetti, P., and Singh, N. (1999). Proteasomal degradation and N-terminal protease resistance of the codon 145 mutant prion protein. *J. Biol. Chem.* 274:23396–23404.
- Zatloukal, K., Stumptner, C., Lehner, M., Denk, H., Baribault, H., Eshkind, L.G., and Franke, W.W. (2000). Cytokeratin 8 protects from hepatotoxicity, and its ratio to cytokeratin 18 determines the ability of hepatocytes to form Mallory bodies. *Am. J. Pathol.* 156:1263–1274.
- Zatloukal, K., Stumptner, C., Fuchsichler, A., Heid, H., Schnoelzer, M., Kenner, L., Kleinert, R., Prinz, M., Aguzzi, A., and Denk, H. (2002). p62 is a common component of cytoplasmic inclusions in protein aggregation diseases. *Am. J. Pathol.* 160:255–263.
- Zeron, M.M., Chen, N., Moshaver, A., Lee, A.T., Wellington, C.L., Hayden, M.R., and Raymond, L.A. (2001). Mutant huntingtin enhances excitotoxic cell death. *Mol. Cell. Neurosci.* 17:41–53.

- Zeron, M.M., Hansson, O., Chen, N., Wellington, C.L., Leavitt, B.R., Brundin, P., Hayden, M.R., and Raymond, L.A. (2002). Increased sensitivity to N-methyl-D-aspartate receptor-mediated excitotoxicity in a mouse model of Huntington's disease [see comment]. *Neuron* 33:849–860.
- Zheng, Y., Wong, M.L., Alberts, B., and Mitchison, T. (1998). Purification and assay of gamma tubulin ring complex. *Methods Enzymol.* 298:218–228.
- Zhou, H., Cao, F., Wang, Z., Yu, Z.X., Nguyen, H.P., Evans, J., Li, S.H., and Li, X.J. (2003). Huntingtin forms toxic NH2-terminal fragment complexes that are promoted by the age-dependent decrease in proteasome activity. *J. Cell Biol.* 163:109–118.
- Zwickl, P., Seemuller, E., Kapelari, B., and Baumeister, W. (2001). The proteasome: a supramolecular assembly designed for controlled proteolysis. *Adv. Protein Chem.* 59:187–222.

Protein Aggregation, Ion Channel Formation, and Membrane Damage

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1. Abstract

A plethora of clinical syndromes are characterized by the deposition of amorphous, Congo red staining material known as “amyloid.” These protein folding diseases include Alzheimer’s, Parkinson’s, type II diabetes mellitus, rheumatoid arthritis, and “mad cow” disease. Amyloid-forming peptides readily adapt beta-sheet structure and can spontaneously aggregate into extended fibrils despite having no primary sequence homology. All amyloid peptides appear to interact strongly with lipid membranes, assemble into oligomers, and form ion-permeable channels. These channels are large, heterogeneous, nonselective, and irreversible. They are inhibited by Congo red and blocked by Zn^{+2} . The leakage pathway induced by these channels could be responsible for the cellular pathology of amyloidoses, including membrane depolarization, mitochondrial dysfunction, inhibition of long-term potential (LTP), and cytotoxicity. We suggest that channel formation underlies amyloid disease.

2. Introduction

Human diseases involving protein misfolding and aggregation have received increasing attention in recent years, partly due to a wealth of new information about the molecular and cellular pathophysiology of these illnesses, and partly due to the increasingly large epidemic of Alzheimer’s disease (AD) and other diseases associated with aging now engulfing the developed world. Over 20 distinct clinical syndromes including AD, Parkinson’s disease (PD), type II diabetes mellitus (DM), rheumatoid arthritis (RA), Creutzfeld-Jakob disease (CJD), and Huntington’s disease (HD) have been associated with misfolded protein aggregates leading to cellular dysfunction and toxicity. The vast majority of these misfolding diseases, known as amyloidoses, are characterized by fibrillar deposits staining with Congo red, and exhibit a classic green birefringence under polarized microscopy (Sipe and Cohen, 2000). The role of these amyloid fibrils in disease was uncertain for many decades, but evidence in recent years has implicated amyloid in the pathogenic process (Merlini and Berlotti, 2003).

Most recently evidence has accumulated to suggest that amyloid fibrils may not be the toxic species, but that smaller aggregates called “oligomers” or “protofibrils” play a crucial role in causing cellular dysfunction and death (Caughey and Lansbury, 2003). Despite this avalanche of new information, the toxic molecular mechanisms of amyloid peptides and proteins have remained obscure. A number of mechanisms have been proposed to account for the toxicity of many peptides. (1) Binding

of amyloid to RAGE (Receptor for Advanced Glycation End products) has been suggested as a possible toxic pathway. (2) Sequestration of vital intracellular factors such as transcription factors, chaperone proteins, cytoskeletal elements, etc., by amyloid aggregates has also been considered (Soto, 2003). (3) A significant body of evidence has pointed to generation of reactive oxygen species (ROS) as a potential pathologic mechanism (Soto, 2003). How amyloid peptides generate ROS, however, is uncertain. (4) Although some evidence has indicated that the preceding mechanisms should be considered, a growing body of experimental evidence points to ion channel formation and membrane damage as the molecular basis of amyloid pathology.

In this chapter we will examine the evidence for membrane damage and channel formation in amyloid diseases, and then go on to discuss cellular membrane-mediated mechanisms by which channel formation could result in pathology. The “channel hypothesis” of amyloidosis appears to explain many of the physiologic effects observed in amyloid diseases, and is consistent with the known properties of amyloid peptides. It proposes a remarkable unity among a variety of seemingly different disorders of human and animal pathology (Table 10-1).

Table 10-1. Protein misfolding diseases

Disease	Protein	Abbreviation
Alzheimer's disease Down's Syndrome (Trisomy 21)	Amyloid precursor protein (Abeta 1-42)	APP (Abeta 1-42)
Heredity cerebral angiopathy (Dutch) Kuru	Prion protein	PrP ^c /PrP ^{sc}
Gerstmann-Straussler Syndrome (GSS) Creutzfeld-Jacob Disease Scrapie (sheep) Bovine spongiform encephalopathy ("mad cow")		
Type II diabetes mellitus (adult onset)	Islet amyloid polypeptide (amylin)	IAPP
Dialysis-associated amyloidosis	Beta-2-microglobulin	2M
Senile cardiac amyloidosis	Atrial natriuretic factor	ANF
Familial amyloid polyneuropathy	Transthyretin	TTR
Reactive amyloidosis familial Mediterranean fever	Serum amyloid A	SAA
Familial amyloid polyneuropathy (Finnish)	Gelsolin	Agel
Macroglobulinemia	Gamma-1 heavy chain	AH
Multiple myeloma	Ig—lambda, Ig—Kappa	AL
Familial polyneuropathy—Iowa (Irish)	Apolipoprotein A1	ApoA1
Hereditary cerebral myopathy—Iceland	Cystatin C	Acys
Nonneuropathic hereditary amyloid with renal disease	Fibrinogen Alpha	AFibA
Nonneuropathic hereditary amyloid with renal disease	Lysozyme	Alys
Familial British dementia	FBDP	A Bri
Familial Danish dementia	FDDP	A Dan
Diffuse Lewy body disease Parkinson's disease	Alpha-synuclein	AS
Fronto-temporal dementia	tau	tau
Amyotrophic lateral sclerosis	Superoxide Dismutase-1	SoD-1
Triplet-repeat diseases (Huntington's, Spinocerebellar ataxias, etc.)	Polyglutamine	PG

3. Alzheimer's Disease (A β)

Alzheimer's disease is a progressive neurodegenerative disorder whose incidence rises exponentially with age. Alzheimer's disease epidemics are sweeping the developed nations of the world whose populations are rapidly aging. Although most AD cases are "sporadic," a number of families with inherited AD have led to the identification of disease causing mutations. The mutated protein, the amyloid precursor protein (APP), is of unknown function. Its metabolism leads to the production of the 42 amino acid peptide Abeta, the prime constituent of amyloid deposits in the brains of AD patients. *In vitro* experiments have demonstrated the cytotoxic potential of Abeta (see Selkoe, 2002, for review), and have specifically indicated that Abeta monomers and fibrils are nontoxic, whereas intermediate states of Abeta aggregation have enhanced toxicity.

Arispe *et al.* (1993a) first demonstrated that Abeta could form ion permeable channels in planar lipid bilayer membranes. These membranes contained only phospholipids, thus indicating the lack of requirement for a specific protein or carbohydrate receptor. The channels described were of several different conductance sizes, consistent with the idea that oligomers/aggregates of varying number and size formed channel structures. The channels obtained were cation selective, but also permeable to divalent cations such as Ca⁺⁺. Zinc ion (Zn⁺⁺), Aluminum ion (Al⁺⁺⁺), and tromethamine (tris⁺) were found to block the channels (Arispe *et al.*, 1993a, 1996). Additionally, very large conductance channels could be observed (4nS), and it was calculated that channels of this size could change the cytosolic [Na⁺] by as much as 10 μ M/sec (Arispe *et al.*, 1993b). The authors proposed that channel formation might be the cytotoxic mechanism of Abeta for neurons. They proposed that Abeta channels would depolarize neuronal membranes by allowing a nonspecific permeability to Na⁺ and Ca⁺⁺. This membrane depolarization could then lead to enhanced Ca⁺⁺ influx through voltage-dependent Ca⁺⁺ channels, and subsequently to cytotoxicity. Because dysregulation of Ca⁺⁺ homeostasis had been observed by others (Mattson *et al.*, 1993), this hypothesis explained some important physiological effects of Abeta. The irregularity of Abeta aggregation was not well understood at this time, however, and efforts to reproduce Abeta channel formation were not always successful (e.g., Mirzabekov *et al.*, 1994) until the importance of Abeta aggregation was better understood and controlled (Hirakura *et al.*, 1999).

Despite these initial difficulties, Abeta induced channel formation or conductance changes were observed in a variety of membranes including neurons (Simmons and Schneider, 1993; Furukawa *et al.*, 1994; Sanderson *et al.*, 1997), oocytes (Fraser *et al.*, 1996), GnRh secreting neurons (Kawahara *et al.*, 1997), liposomes (Lin *et al.*, 1999), and planar lipid bilayer membranes (Hirakura *et al.*, 1999; Kourie *et al.*, 2001). It has also been observed that Abeta, short of killing cells, can inhibit LTP, and that only channel-forming variants of the Abeta peptide have LTP inhibiting activity (Chen *et al.*, 2000). However, this study, which employed a shortened form of Abeta, Abeta 25–35, also showed that certain channel forming peptides were *not* cytotoxic and did not inhibit LTP, suggesting that channel formation is necessary for Abeta pathology, but not sufficient to explain it.

Abeta can also decrease synaptic efficiency *in vivo*. For example, the V717F APP mouse exhibits decreased excitatory postsynaptic potential and fast decay of LTP at age 4–5 months (Larson *et al.*, 1999). Another APP transgenic mutant (V642I) showed LTP decay at age 5–7 months (Moechars *et al.*, 1999). These electrophysiology deficits appear to be directly related to increased Abeta production (Hsia *et al.*, 1999). Evidence using immunodepletion or proteolytic degradation suggests that Abeta oligomers are responsible for these alterations in synaptic functions (Walsh *et al.*, 2002).

Additionally, it has been shown that Abeta can kill fibroblasts by forming channels in them. Toxicity is blocked by Zn⁺² at concentrations that block Abeta channels (Zhu *et al.*, 2000). These

investigators also showed that Abeta could mediate Ca^{++} transport into liposomes (Lin *et al.*, 1999). Kourie *et al.* (2002) has characterized at least three distinct Abeta channel types in planar lipid bilayers and shown that Ca^{++} can modify channel kinetics (Bahadi *et al.*, 2003a). Hirakura *et al.* (1999) showed that monomerization of Abeta in organic solvents abolished channel activity, but that activity returned as the peptide reaggregated in aqueous solution. Single-channel conductance also increased with time of aggregation, suggesting that Abeta aggregated into progressively larger oligomers with increasingly large sizes and channel conductances (see Table 10-2). This heterogeneity of single-channel conductances has been seen in all electrophysiologic studies of Abeta to date, and seems to reflect the heterogeneity of the Abeta aggregation process, with many oligomeric species of varying sizes present in solution at a given moment.

Table 10-2. Channel properties of amyloid peptides

Peptide	Single channel conductance	Ion selectivity (permeability ratio)	Blockade by zinc	Inhibition by Congo Red	Reference
A β 25–35	10–400 pS	Cation ($P_K/P_{Cl} = 1.6$)	+	+	Mirzabekov <i>et al.</i> (1994)
A β 1–40	10–2000 pS	Cation ($P_K/P_{Cl} = 1.8$)	+	N.D.	Lin and Kagan (2002)
A β 1–40	50–4000 pS	Cation ($P_K/P_{Cl} = 11.1$)	+	N.D.	Hirakura <i>et al.</i> (1999)
A β 1–42	10–2000 pS	Cation ($P_K/P_{Cl} = 1.8$)	+	+	Arispe <i>et al.</i> (1993a, 1993b, 1996)
CT105 (C-terminal fragment of amyloid precursor protein (APP))	120 pS	Cation	+	+	Hirakura <i>et al.</i> (1999)
Islet amyloid polypeptide (amylin)	7.5 pS	Cation ($P_K/P_{Cl} = 1.9$)	+	+	Kim <i>et al.</i> (1999)
PrP106–126	10–400 pS	Cation ($P_K/P_{Cl} = 2.5$)	+	+	Mirzabekov <i>et al.</i> (1996)
PrP106–126	140,900, 1444 pS	Cation ($P_K/P_{Cl} > 10$)	N.D.	N.D.	Lin <i>et al.</i> (1997)
PrP 82–146		Cation (variable)	N.D.	N.D.	Kourie and Culverson (2000)
Serum amyloid A	10–1000 pS	Cation ($P_K/P_{Cl} = 2.9$)	+	+	Bahadi <i>et al.</i> (2003b)
C-type natriuretic peptide	21, 63 pS	Cation ($P_K/P_{Cl} > 10$)	+	+	Hirakura <i>et al.</i> (2002)
Atrial natriuretic factor	68, 160, 273 pS	Various	N.D.	N.D.	Kourie (1999)
Beta2-Microglobulin	0.5–120 pS	Non-selective	+	+	Kourie <i>et al.</i> (2001)
Transthyretin	Variable	Cation (Variable)	+	+	Hirakura and Kagan (2001)
Polyglutamine	19–220 pS	Non-selective	–	–	Hirakura <i>et al.</i> (2001)
Polyglutamine	17 pS	Cation	N.D.	N.D.	Hirakura <i>et al.</i> (2000)
NAC (alpha-synuclein 65–95)	10–300 pS	Variable	+	+	Mono <i>et al.</i> (2000)
Calcitonin					Azimova <i>et al.</i> (2003)
human	12 pS	N.D.	N.D.	N.D.	Stipani <i>et al.</i> (2001)
salmon	580	Non-selective	N.D.	N.D.	

N.D. —not determined.

The sensitivity of channel-forming activity to lipid composition of the membrane was reported by Lin and Kagan (2002) for the Abeta 25–35 peptide. Negatively charged lipids enhanced channel activity, while the presence of cholesterol inhibited channel activity. The relevance of this finding to *in vivo* Abeta cytotoxicity was demonstrated by Arispe and Doh (2002), who showed that Abeta cytotoxicity was increased by agents that decreased plasma membrane cholesterol concentrations and decreased by agents that increased membrane cholesterol. However, it should be noted that another group has found that sterols appear to enhance Abeta channel formation (Micelli *et al.*, 2004). The reason for these discrepant findings is uncertain, but we must always be concerned that the aggregation state of Abeta used is the same in both experiments. Unfortunately, aggregation state was not directly measured in either report.

Arispe (2004) has gone on to show that the Abeta pore is blocked by peptides hypothesized to bind to a putative pore lining sequence. This lends some credence to one of the proposed structural models for the Abeta pore (Durell *et al.*, 1994). It also confirms the probable role of a histidine pair binding Zn^{+2} at the mouth of the pore.

Confirmatory observations using electron microscopy have shown that protofibrils of Abeta can form “annular” structures. Although these structures are occurring in the absence of lipid membranes and are likely too large to be the same as the channels observed in physiology experiments, their presence is suggestive that aggregation into extended fibrils is not the only pathologic conformation open to Abeta monomers. It is also noteworthy that mutant Abeta peptides associated with familial AD show an increased tendency to form these annular structures (Lashuel *et al.*, 2002).

4. Prion (PrP) Channels

Prions are infectious proteinaceous particles responsible for transmissible spongiform encephalopathies in animals (scrapie, “mad cow” disease) and humans (CJD, Kuru, Gerstmann-Straussler-Scheinker (GSS) syndrome). These neurodegenerative diseases are caused by the transition of the cellular prion protein (PrP^c) to a toxic, infectious form (PrP^{Sc}). The transition is characterized by loss of alpha-helix and an increase in beta-sheet content. PrP^{Sc} often becomes insoluble and protease-resistant. It can aggregate into amyloid fibrils that bind Congo red (Pan *et al.*, 1993). Forloni *et al.* (1993) reported that a PrP fragment, PrP 106–126, was neurotoxic. Lin *et al.* (1997) showed that PrP106–126 could form ion channels in planar lipid bilayer membranes. These channels, like those of Abeta, were long lived, irreversible, relatively nonselective, heterogeneous in terms of single-channel conductances, inhibited from forming by Congo red, and blocked by Zn^{+2} .

The remarkable similarity of PrP106–126 and Abeta channels led Lin *et al.* (1997) to suggest that channel formation was a pathological cellular mechanism common to amyloid diseases. Aging in solution of the PrP106–126 peptide, which promotes aggregation, enhanced channel-forming activity by as much as two orders of magnitude. Exposure to acidic pH (4.5), which also promotes aggregation, enhanced channel activity by 50-fold and caused an upward shift in the observed distribution of single-channel conductances. Acidic pH is also known to promote the transition of PrP106–126 from alpha-helical conformation to beta-sheet (De Gioia *et al.*, 1994). This transition is critical in the shift of cellular PrP to the pathologic scrapie form.

Kourie and Culverson (2000) confirmed the channel-forming activity of PrP106–126 and demonstrated that several distinct cation channels were reproducibly formed by this peptide. They reported a tetraethylammonium-sensitive channel with fast kinetics (140 pS), a dithiodipyridin-

sensitive channel with slow kinetics (40 pS), and a 900–1500 pS channel with subconductance levels. This group further showed that the antimalarial drug quinacrine blocked PrP106–126 channels by reducing the average channel current through the open pore (Farrelly *et al.*, 2003), a finding that correlates with the ability of quinacrine to inhibit prion propagation (Ryou *et al.*, 2003).

Further experiments showed that copper ion (Cu^{+2}) could alter channel properties (Kourie *et al.*, 2003). Most recently, this group has reported channel formation by a larger prion protein fragment, PrP80–146 (Bahadi *et al.*, 2003b). This fragment is found in the amyloid deposits in the brains of patients with GSS disease. The channel properties are nearly identical to those of PrP106–126. Furthermore, scrambling the 127–146 sequence does not affect channel activity of the PrP80–146, whereas scrambling the 106–126 sequence abolishes channel activity. Thus, the 106–126 sequence appears to be the critical channel-forming region in the larger prion protein.

Although amyloid deposits are found in most prion disease, it is not universal. An interesting exception is a familial form of prion disease where PrP becomes a transmembrane protein (Hegde *et al.*, 1998). It is tempting to speculate that the 106–126 sequence may be forming a channel in this prion disease.

Further evidence for the channel hypothesis comes from a recent study showing that scrapie infected GT1–1 cells exhibit impaired function of voltage dependent N-type calcium channels. This impairment is reversed by quinacrine, a prion channel blocker (Sandberg *et al.*, 2004).

5. Type II Diabetes Mellitus and Islet Amyloid Polypeptide (IAPP, Amylin)

Adult-onset diabetes mellitus (type II) is characterized by initial resistance to the effects of insulin followed by loss of insulin secreting capacity. This loss is concurrent with a reduction in the number of insulin secreting beta-cells in the pancreas (Westermarck and Vilender, 1978). Islet amyloid polypeptide (IAPP, Amylin), a 37-amino acid hormone, is cosecreted with insulin. IAPP forms amyloid deposits in the pancreas of type II diabetics, and its presence is correlated with the loss of beta-cells. Mice transgenic for IAPP developed loss of beta-cells with diabetes (Butler *et al.*, 2003). Alzheimer's disease patients are at increased risk for type II diabetes, suggesting a link between the two disease processes (Janson *et al.*, 2004).

Islet amyloid polypeptide exhibits toxicity to beta-cells in culture (Lorenzo *et al.*, 1994). Islet amyloid polypeptide is able to interact strongly with planar lipid bilayer membranes (Mirzabekov *et al.*, 1996). Channel formation is voltage-independent, irreversible, and shows a nonlinear dependence on peptide concentration, suggesting that at least three IAPP molecules come together to form the channel structure. Single-channel conductances are uniform (7.5 pS in 10 mM KCl) in contrast to those of other amyloid channels. Channels can be closed with voltage once inserted, and only reopen slowly at permissive voltages. Human IAPP forms channels at cytotoxic concentrations, whereas the non-toxic and nonamyloidogenic rat IAPP does not form channels. Rat IAPP differs from human IAPP at five amino acid positions. Rats do not suffer from type II diabetes mellitus.

Channel-forming activity could be inhibited by increasing concentrations of salt in the solution, suggesting an effect of negative lipid surface charge on the positively charged (+5) IAPP. Experiments that showed that increasing negative surface charge increased IAPP channel activity were consistent with this idea. Addition of cholesterol, which increased membrane fluidity, caused a decrease in IAPP channel activity.

Human IAPP fibrils have been shown to be nontoxic, whereas smaller aggregates possess toxicity (Janson *et al.*, 1999). The smaller aggregates, but not fibrils, were shown to have the ability to disrupt lipid bilayer membranes. The size of these aggregates varied between 25–6000 molecules as

measured by light-scattering. Islet amyloid polypeptide insertion into lipid monolayers and bilayers could be inhibited by Congo red and arrested by rifampicin despite continued formation of amyloid (Harroun *et al.*, 2001). This implies that nonfibrillar IAPP is the critical cytotoxic species. “Protofibrillar” IAPP was reported to permeabilize liposomes to Ca^{+2} , while not permitting fura-2 (MW 832) or FITC-Dextran (MW 4400) to escape (Anguiano *et al.*, 2002). This suggests a relatively narrow pore region consistent with the low single-channel conductance reported by Mirzabekov *et al.* (1996). Monomeric IAPP lacked channel-forming activity, but channel activity returned as IAPP was allowed to aggregate in aqueous solution (Hirakura *et al.*, 2000). Islet amyloid polypeptide has also been observed to disrupt calcium homeostasis in neurons (Kawahara *et al.*, 2000) similar to the effects of Abeta and PrP106–126. The ability of Congo red to inhibit IAPP channel formation also is similar to results with Abeta and PrP106–126 (Hirakura *et al.*, 2000). Finally Zn^{+2} , which reversibly blocks Abeta and PrP106–126 channels, exhibits a similar blockade on IAPP channels.

6. α -Synuclein and Parkinson’s Disease

Parkinson’s disease is a progressive neurodegenerative disorder characterized by bradykinesia, rigidity, and tremor. Pathologically, the illness is characterized by loss of dopaminergic neurons in the substantia nigra and the presence of Lewy bodies. Lewy bodies are intracellular inclusions consisting primarily of aggregated (fibrillar) alpha-synuclein (AS). Mutations in the AS protein (A30P, A53T) can lead to inherited PD.

Alpha-synuclein is a synaptic protein of unknown function that is associated with synaptic vesicles. A hydrophobic peptide from the central region of AS, AS60–95, is found in the amyloid deposits of AD. There is considerable clinical overlap between the syndromes of AD and PD. For example, some AD patients exhibit motor abnormalities. Parkinson’s disease patients often suffer from dementia in addition to motor impairments. Intermediate syndromes such as “dementia with Lewy bodies” imply that AS may be damaging to neurons other than the dopaminergic cells targeted in PD (McKeith *et al.*, 2004). *Drosophila* models of PD (transgenic for AS mutants) exhibit a movement disorder, degeneration of dopaminergic neurons, and fibrillar inclusions. Mouse models show similar but less reproducible defects.

Alpha-synuclein can permeabilize liposomes to solutes in a graded fashion according to molecular weight (Volles and Lansbury, 2002). This implies a “pore-like” mechanism. Parkinson’s disease inducing mutations such as A53T and A30P accelerate both the formation of AS oligomers and the permeabilization activity of AS. The hydrophobic central peptide AS60–95, known as a NAC (nonamyloid component), is key to the oligomerization/fibrillation of AS. Nonamyloid component forms channels in planar lipid bilayer membranes that are strikingly reminiscent of the channels formed by other amyloids such as Abeta and PrP106–126. Single-channel conductances of NAC are heterogeneous. The channels are long lived, nonselective, inhibited by Congo red and blocked by Zn^{+2} (Kagan and Azimova, 2003). Alpha-synuclein can also form annular or “ring-like” structures as viewed by electron microscopy (Lashuel *et al.*, 2002). Parkinson’s disease mutations accelerate the formation of these structures.

7. Polyglutamine and Triplet Repeat Diseases

Huntington’s disease is an autosomal dominant, progressive neurodegenerative disorder characterized by choreiform movements and psychiatric symptoms. It is but one of a group of “triplet repeat diseases,” caused by a pathological expansion of the codon CAG, which codes for the amino

acid glutamine. In each illness there is a critical repeat length, beyond which the subject experiences the disease symptoms. For HD, the critical length is 37 amino acids. This leads to a toxic “gain of function” in the protein *huntingtin*, whose function remains obscure. The length of the polyglutamine (PG) tract is inversely correlated with age of onset of illness in Huntington’s disease, suggesting that PG may play a pathological role in the disease (Li and Li, 2004). Huntington’s disease is characterized by neuronal loss in striatum and cortex, but earlier manifestations of the illness may include memory deficits, behavioral abnormalities, and impairment of LTP, a model for memory. Although triplet repeat diseases are not true amyloid diseases, they are characterized by aggregation of polyglutamine containing proteins into destructive oligomers. The formation of aggregates correlates in HD animal models with disease progression. In cell culture (PC12), expression of *huntingtin* with a polyglutamine length of 150 increases vulnerability to apoptosis even without aggregate formation.

Channel formation by PG has been reported by two groups: Monoi *et al.* (2000) and Hirakura *et al.* (2000). The latter group observed channels that were long-lived, relatively nonselective among physiologic ions, and heterogeneous (single-channel conductances of 19–220 pS in 0.1 M KCl). They employed a mixture of polyglutamine species (average MW = 6000), which is consistent with the heterogeneity they observed. Acidic pH enhanced channel formation. Congo red did not inhibit channel formation, and Zn⁺² did not block channels, unlike other “true” amyloid peptides. These findings clearly distinguish the CAG repeat illnesses from classic amyloidoses. Monoi *et al.* (2000) observed a homogenous set of channels that were cation selective with a uniform single-channel conductance of 17 pS in 1 M CsCl. They employed a single species of PG with repeat length of 40.

A similar PG preparation of repeat length 29 failed to form channels consistent with the HD critical repeat length of 37. They proposed a model, the mu-helix, for the PG-induced channel. The minimum membrane spanning length for a mu-helix is 37 residues, again consistent with the critical length determined for human pathology.

Other experiments implicate mitochondria as the site of damage in HD. Panov *et al.* (2002) observed decreased membrane potential and increased susceptibility to depolarization in HD mitochondria compared to controls. Similar findings were seen in brain mitochondria of transgenic mice expressing *huntingtin* with a pathogenic PG tract. *Huntingtin* could be observed by electron microscopy on mitochondrial membranes. It is of interest that a non-*huntingtin* fusion protein with an extended PG tract showed similar results, implicating PG itself in mitochondrial membrane pathology. These results strongly suggest that PG can damage mitochondrial membranes by a channel mechanism.

8. Mechanisms of Membrane-Mediated Damage

8.1. Plasma Membranes

Membrane-mediated cytotoxicity is well documented in the microbial world, but is less well understood in higher eukaryotes and especially neurons. Because bacteria use their (inner) cell membrane for respiration and active transport, permeability changes in this barrier are poorly tolerated. Furthermore, the relatively small volume of bacterial cells makes them vulnerable to loss of vital intracellular ions such as K⁺ and Mg⁺⁺, and influx of toxic extra cellular ions such as Ca⁺⁺. The cytotoxicity of channel-forming antimicrobial agents has been clearly linked with their ability to breach the permeability barrier of the cell membrane (e.g., Schein *et al.*, 1978; Kagan *et al.*, 1990;

Sokolov *et al.*, 1999). Eukaryotic cells are somewhat protected from these agents due to their larger volume and the presence of sterols in the plasma membrane, which strongly reduces the ability of most toxins to penetrate the membrane. A notable exception is the antifungal drug and channel-former nystatin that not only kills fungi, but also afflicts mammalian host cells causing considerable side effects and toxicity (Ng *et al.*, 2003). This clearly demonstrates that channel formation alone can damage and kill mammalian cells. The vulnerability of different cell types depends on their specialization, including factors such as membrane fluidity, membrane potential, ionic pumping capability, etc.

The cholesterol-dependent cytolysins (e.g., perfringolysin O of *Clostridium*) are a family of bacterial pore-forming toxins that also require cholesterol for pore formation. These toxins play a key role in *Clostridial* pathogenesis by punching large pores in the host mammalian cell membrane. It is noteworthy that similar to amyloid peptides these toxins form pores by the membrane-mediated conversion of alpha-helical regions to beta-sheet structure (Ramachandran *et al.*, 2004). Each toxin monomer appears to contribute two beta-sheet hairpins to the pore. Although the structure of these pores is not yet known, they are known to be oligomeric, with up to 50 monomers contributing to a pore up to 300 Angstroms in diameter. The staphylococcal alpha-hemolysin forms a heptameric pore with each subunit contributing two beta-strands to a 14-strand beta-barrel pore (Song *et al.*, 1996). The porin pores of Gram-negative bacterial outer membranes are also known to have a beta-barrel structure (Cowan *et al.*, 1992). Thus, the beta-barrel plays a key role in pore structures of toxins and bacterial membrane proteins.

Neurons are particularly vulnerable to insults to plasma membrane integrity. Neurons rely on plasma membrane potential to transmit signals in the nervous system. This requires a plasma membrane of low baseline ionic permeability. Any breach in this permeability requires the neurons to pump ions ($\text{Na}^+/\text{Ca}^{++}$ out and K^+ in) faster at a higher metabolic cost. Leakage pathways in the membrane depolarize the membrane and lead to defective signaling. Nonselective channels may also give access to influx of ions like Ca^{++} , whose intracellular concentration is highly regulated at considerable metabolic expense. Increased levels of intracellular Ca^{++} can activate numerous metabolic pathways including that of apoptosis. Depolarization of the plasma membrane by leakage channels can also lead to influx of Ca^{++} through voltage-dependent Ca^{++} channels. These physiologic functions, taken together, suggest that the plasma membrane would be a vulnerable target for irreversible, non-specific channels such as those formed by amyloid proteins. It is perhaps not surprising that neuropathology is so common in amyloid diseases because neurons have so many physiologic vulnerabilities and depend so critically on intact plasma membranes.

8.2. Mitochondrial Membranes

Although the plasma membrane seems to be the logical place for damage by amyloid channels to occur, substantial evidence implicates mitochondrial membranes as a possible target. Mitochondria originated as bacterial symbionts, and their membranes reflect this origin. The outer membrane is relatively permeable to small solutes through aqueous pores (mitochondrial porins or VDAC), and the inner membrane is relatively impermeable to allow the coupling of respiration to oxidative phosphorylation via the generation of an H^+ gradient across the membrane. The appearance of the permeability transition pore (PTP) in the inner membrane signals depolarization, efflux of cytochrome *c*, and apoptosis. Many apoptosis proteins such as bax and Bcl-2 are channel formers with structural similarities to the channel forming bacterial toxins known as colicins (Schein *et al.*, 1978; Reed, 2000). The frequency of increased apoptosis in amyloid diseases suggest that amyloid proteins might act on mitochondria to increase PTP formation and apoptosis, perhaps through a direct pore-forming

effect. Abeta has been shown to depolarize mitochondria and to lead to PTP formation and cytochrome *c* release (Rodriguez *et al.*, 2000; Parks, *et al.*, 2001; Kim *et al.*, 2002). Many amyloid peptides increase reactive oxygen species, and this effect is likely mediated through mitochondria. Polyglutamine can directly depolarize and damage mitochondria, and HD mitochondria appear to be damaged even prior to observable disease or pathology (Panov *et al.*, 2002). Calcium homeostasis is dysregulated by amyloid peptides, and mitochondria are an important regulator of Ca^{++} stores. Abeta peptides have been shown to induce directly mitochondrial dysfunction, oxidative stress, and loss of mitochondrial membrane potential. Abeta causes a series of Ca^{++} -dependent depolarizations, leading to a gradual collapse of potential. The slow collapse, but not the Ca^{++} -dependent depolarizations, can be blocked by antioxidants. This suggests that Abeta channel formation leads to mitochondrial membrane depolarizations followed by an increase in oxygen radical production. These radicals can lead to overall membrane potential loss and apoptosis (Abramov *et al.*, 2004). When combined together, these data provide a compelling case that mitochondrial membranes may be an important target of amyloid peptide damage.

8.3. Other Intracellular Membranes

The accumulation of Abeta in endosome/lysosome/proteosomal components led to the suggestion that permeabilization of these membranes could lead to cellular pathology (Knauer *et al.*, 1992). Since this observation, however, little evidence has been gathered to implicate this pathway. It certainly seems likely that a defect in these membranes would lead to cellular dysfunction and death, but direct evidence is not presently available. Other intracellular membranes such as Golgi, endoplasmic reticulum, peroxisomes, etc., have been considered as potential targets, but it is unclear how channel formation would lead to cellular dysfunction in these cases.

8.4. Other Amyloid Peptides

Several other amyloid peptides have been found to form ion channels. These include atrial natriuretic factor (ANF, Kourie, 1999), beta-2-microglobulin (B2M, Hirakura and Kagan, 2001), serum amyloid A (SAA, Sipe, 2000; Hirakura *et al.*, 2002; Lashuel *et al.*, 2003), and transthyretin (Hirakura *et al.*, 2002). All of these channels exhibit remarkably similar properties. The channels are heterogeneous, long-lived, irreversible, nonselective, inhibited by Congo red and blocked by Zn^{+2} . It has also been shown that proteins not associated with amyloid diseases such as HypF, the N-terminal domain of the *Escherichia coli* hydrogenase mutation factor, can aggregate into amyloid like fibrils under destabilizing conditions (Relini *et al.*, 2004). Prefibrillar aggregates of HypF can permeabilize liposomes, whereas fibrils cannot. Furthermore, these aggregates can cause Ca^{++} disregulating oxygen free radical production and cytotoxicity similar to that caused by prefibrillar aggregates of disease associated amyloid peptides (Bucciantini *et al.*, 2004). This suggests that the misfolded beta-sheet conformation leads to aggregation and membrane insertion in a wide variety of proteins, pathologic and nonpathologic. These findings strongly suggest that the nature of protein misfolding and aggregation is deeply connected to the physical chemistry of conversion of an aqueous protein into a membrane penetrating and pore-forming structure. The adaptability of protein conformation, which allows the transport and trafficking of cellular proteins, also has the potential to allow soluble native proteins to become pathologic pores under the appropriate conditions. Further research should elucidate the detailed molecular mechanisms of these remarkable conformational changes.

9. Abbreviations

AD	Alzheimer's disease
APP	Amyloid precursor protein
AS	Alpha-synuclein
CJD	Creutzfeld-Jakob disease
DM	Type II diabetes mellitus
HD	Huntington's disease
IAPP	Islet amyloid polypeptide
LTP	Long-term potentiation
PD	Parkinson's disease
PG	Polyglutamine
PrP	Prion protein
PTP	Permeability transition pore
RA	Rheumatoid arthritis
RAGE	Receptor for Advanced Glycation End products
ROS	Reactive oxygen species

References

- Abramov, A.Y., Canevari, L., and Duchen, M.R. (2004). Beta-amyloid peptides induce mitochondrial dysfunction and oxidative stress in astrocytes and death of neurons through activation of NADPH oxidase. *J. Neurosci.* 24:565–575.
- Anguiano, M., Nowak, R.J., and Lansbury, P.T., Jr. (2002). Protofibrillar islet amyloid polypeptide permeabilizes synthetic vesicles by a pore-like mechanism that may be relevant to type II diabetes. *Biochemistry* 41(38):11338–11343.
- Arispe, N. (2004). Architecture of the Alzheimer's A beta P ion channel pore. *J. Membr. Biol.* 197:33–48.
- Arispe, N., and Doh, M. (2002). Plasma membrane cholesterol controls the cytotoxicity of Alzheimer's disease A beta P (1–40) and (1–42) peptides. *FASEB J.* 16:1526–1536.
- Arispe, N., Rojas, E., and Pollard, H.B. (1993a). Alzheimer disease amyloid beta protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminum. *Proc. Natl. Acad. Sci. USA* 90(1):567–571.
- Arispe, N., Pollard, H.B., and Rojas, E. (1993b). Giant multilevel cation channels formed by Alzheimer disease amyloid beta-protein [A beta P-(1–40)] in bilayer membranes. *Proc. Natl. Acad. Sci. USA* 90:10573–10577.
- Arispe, N., Pollard, H.B., and Rojas, E. (1996). Zn²⁺ interaction with Alzheimer amyloid beta protein calcium channels. *Proc. Natl. Acad. Sci. USA* 93:1710–1715.
- Bahadi, R., Farrelly, P.V., Kenna, B.L., Curtain, C.C., Masters, C.L., Cappai, R., Barnham, K.J., and Kourie, J.I. (2003a). Cu²⁺-induced modification of the kinetics of A beta(1–42) channels. *Am. J. Physiol.* 285:C873–C880.
- Bahadi, R., Farrelly, P.V., Kenna, B.L., Kourie, J.I., Tagliavini, F., Forloni, G., and Salmons, M. (2003b). Channels formed with a mutant prion protein PrP(82–146) homologous to a 7-kDa fragment in diseased brain of GSS patients. *Am. J. Physiol.* 285:C862–C872.
- Bucciantini, M., Calloni, G., Chiti, F., Formigli, L., Nosi, D., Dobson, C.M., and Stefani, M. (2004). Prefibrillar amyloid protein aggregates share common features of cytotoxicity. *J. Biol. Chem.* 279:31374–31382.
- Butler, A.E., Janson, J., Soeller, W.C., and Butler P.C. (2003). Increased beta-cell apoptosis prevents adaptive increase in beta-cell mass in mouse model of type 2 diabetes: evidence for role of islet amyloid formation rather than direct action of amyloid. *Diabetes* 52:2304–2314.
- Caughey, B., and Lansbury, P.T., Jr. (2003). Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders. *Annu. Rev. Neurosci.* 26:267–298.
- Chen, Q.S., Kagan, B.L., Hirakura, Y., and Xie, C.W. (2000). Impairment of hippocampal long-term potentiation by Alzheimer amyloid beta-peptides. *J. Neurosci. Res.* 60:65–72.
- Cowan, S.W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R.A., Jansonius, J.N., and Rosenbusch, J.P. (1992). Crystal structures explain functional properties of two *E. coli* porins. *Nature* 358:727–733.

- De Gioia, L., Selvaggini, C., Ghibaudi, E., Diomed, L., Bugiani, O., Forloni, G., Tagliavini, F., and Salmona, M. (1994). Conformational polymorphism of the amyloidogenic and neurotoxic peptide homologous to residues 106–126 of the prion protein. *J. Biol. Chem.* 269:7859–7862.
- Durell, S.R., Guy, H.R., Arispe, N., Rojas, E., and Pollard, H.B. (1994). Theoretical models of the ion channel structure of amyloid beta-protein. *Biophys. J.* 67:2137–2145.
- Farrelly, P.V., Kenna, B.L., Laohachai, K.L., Bahadi, R., Salmona, M., Forloni, G. and Kourie, J.I. (2003). Quinacrine blocks PrP (106–126)-formed channels. *J. Neurosci. Res.* 74:934–941.
- Forloni, G., Chiesa, R., Smiroldo, S., Verga, L., Salmona, M., Tagliavini, F., and Angeretti, N. (1993). Apoptosis mediated neurotoxicity induced by chronic application of beta amyloid fragment 25–35. *Neuroreport* 4:523–526.
- Fraser, S.P., Suh, Y.H., Chong, Y.H., and Djamgoz, M.G. (1996). Membrane currents induced in *Xenopus* oocytes by the C-terminal fragment of the beta-amyloid precursor protein. *J. Neurochem.* 66(5):2034–2040.
- Furukawa, K., Abe, Y., and Akaike, N., (1994). Amyloid beta protein-induced irreversible current in rat cortical neurones. *Neuroreport* 5:2016–2018.
- Harroun, T.A., Bradshaw, J.P., and Ashley, R.H. (2001). Inhibitors can arrest the membrane activity of human islet amyloid polypeptide independently of amyloid formation. *FEBS Lett.* 507:200–204.
- Hegde, R.S., Mastrianni, J.A., Scott, M.R., DeFea, K.A., Tremblay, P., Torchia, M., DeArmond, S.J., Prusiner, S.B., and Lingappa, V.R. (1998). A transmembrane form of the prion protein in neurodegenerative disease. *Science* 279:827–834.
- Hirakura, Y., and Kagan, B.L. (2001). Pore formation by beta-2-microglobulin: a mechanism for the pathogenesis of dialysis associated amyloidosis. *Amyloid* 8:94–100.
- Hirakura, Y., Lin, M.C., and Kagan, B.L. (1999). Alzheimer amyloid abeta1–42 channels: effects of solvent, pH, and Congo Red. *J. Neurosci. Res.* 57:458–466.
- Hirakura, Y., Azimov, R., Azimova, R., and Kagan, B.L. (2000). Polyglutamine-induced ion channels: a possible mechanism for the neurotoxicity of Huntington and other CAG repeat diseases. *J. Neurosci. Res.* 60:490–494.
- Hirakura, Y., Azimova, R., Azimov, R., and Kagan, B.L. (2001). Ion channels with different selectivity formed by transthyretin. *Biophys. J.* 80–120.
- Hirakura, Y., Carreras, I., Sipe, J.D., and Kagan B.L. (2002). Channel formation by serum amyloid A: a potential mechanism for amyloid pathogenesis and host defense. *Amyloid* 9:13–23.
- Hsia, A.Y., Masliah, E., McConlogue, L., Yu, G.Q., Tatsuno, G., Hu, K., Kholodenko, D., Malenka, R.C., Nicoll, R.A., and Mucke, L. (1999). Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. *Proc. Natl. Acad. Sci. USA* 96:3228–3233.
- Janson, J., Ashley, R.H., Harrison, D., McIntyre, S., and Butler, P.C. (1999). The mechanism of islet amyloid polypeptide toxicity is membrane disruption by intermediate-sized toxic amyloid particles. *Diabetes* 48:491–498.
- Janson, J., Laedtke, T., Parisi, J.E., O'Brien P., Petersen R.C., and Butler, P.C. (2004). Increased risk of type 2 diabetes in Alzheimer disease. *Diabetes* 53:474–481.
- Kagan, B.L., Selsted, M.E., Ganz, T., and Lehrer, R.I. (1990). Antimicrobial defensin peptides form voltage dependent ion permeable channels in planar lipid bilayer membranes. *Proc. Natl. Acad. Sci. USA* 87:210–214.
- Kagan, B.L., Azimova, R.K. (2003). Ion channels formed by a fragment of alpha-synuclein (NAC) in lipid membranes. *Biophys. J.* 84(2):53a.
- Kawahara, M., Arispe, N., Kuroda, Y., and Rojas, E. (1997). Alzheimer's disease amyloid beta-protein forms Zn(2+)-sensitive, cation-selective channels across excised membrane patches from hypothalamic neurons. *Biophys. J.* 73:67–75.
- Kawahara, M., Kuroda, Y., Arispe, N., and Rojas, E. (2000). Alzheimer's beta-amyloid, human islet amylin, and prion protein fragment evoke intracellular free calcium elevations by a common mechanism in a hypothalamic GnRH neuronal cell line. *J. Biol. Chem.* 275:14077–14083.
- Kim H.J., Suh, Y.H., Lee, M.H., and Ryu, P.D., (1999). Cation selective channels formed by a C-terminal fragment of beta-amyloid precursor protein. *Neuroreport* 14, (10):1427–1431.
- Kim, H.S., Lee, J.H., Lee, J.P., Kim, E.M., Chang, K.A., Park, C.H., Jeong, S.J., Wittendorp, M.C., Seo, J.H., Choi, S. H., and Suh, Y.H. (2002). Amyloid beta peptide induces cytochrome *c* release from isolated mitochondria. *Neuro-report* 13:1989–1993.
- Knauper, M.F., Soreghan, B., Burdick, D., Kosmoski, J., and Glabe, C.G. (1992). Intracellular accumulation and resistance to degradation of the Alzheimer amyloid A4/beta protein. *Proc. Natl. Acad. Sci. USA* 89:7437–7441.
- Kourie, J.I. (1999). Characterization of a C-type natriuretic peptide (CNP-39)-formed cation-selective channel from platypus (*Ornithorhynchus anatinus*) venom. *J. Physiol.* 518(Pt 2):359–369.
- Kourie, J.I., and Culverson, A. (2000). Prion peptide fragment PrP[106–126] forms distinct cation channel types. *J. Neurosci. Res.* 62:120–133.

- Kourie, J.I., Henry, C.L., and Farrelly, P. (2001). Diversity of amyloid beta protein fragment [1–40]-formed channels. *Cell. Mol. Neurobiol.* 21:255–284.
- Kourie, J.I., Culverson, A.L., Farrelly, P.V., Henry, C.L., and Laohachai, K.N. (2002). Heterogeneous amyloid-formed ion channels as a common cytotoxic mechanism: implications for therapeutic strategies against amyloidosis. *Cell Biochem. Biophys.* 36:191–207.
- Kourie, J.I., Kenna, B.L., Tew, D., Jobling, M.F., Curtain, C.C., Masters, C.L., Barnham, K.J., and Cappai, R. (2003). Copper modulation of ion channels of PrP[106–126] mutant prion peptide fragments. *J. Membr. Biol.* 193:35–45.
- Larson, J., Lynch, G., Games, D., and Seubert, P. (1999). Alterations in synaptic transmission and long-term potentiation in hippocampal slices from young and aged PDAPP mice. *Brain Res.* 840:23–35.
- Lashuel, H.A., Petre, B.M., Wall, J., Simon, M., Nowak, R.J., Walz, T., and Lansbury, P.T., Jr. (2002). Alpha-synuclein, especially the Parkinson's disease-associated mutants, forms pore-like annular and tubular protofibrils. *J. Mol. Biol.* 322:1089–1102.
- Lashuel, H.A., Hartley, D.M., Petre, B.M., Wall, J.S., Simon, M.N., Walz, T., and Lansbury, P.T., Jr. (2003). Mixtures of wild-type and a pathogenic (E22G) form of Abeta40 in vitro accumulate protofibrils, including amyloid pores. *J. Mol. Biol.* 332:795–808.
- Li, S.H., and Li, X.J. (2004). Huntingtin–protein interactions and the pathogenesis of Huntington's disease. *Trends Genet.* 20:146–154.
- Lin, M.C., and Kagan, B.L. (2002). Electrophysiologic properties of channels induced by Abeta25–35 in planar lipid bilayers. *Peptides* 23:1215–1228.
- Lin, M.C., Mirzabekov, T., and Kagan, B.L. (1997). Channel formation by a neurotoxic prion protein fragment. *J. Biol. Chem.* 272:44–47.
- Lin, H., Zhu, Y.J., and Lal, R. (1999). Amyloid beta protein (1–40) forms calcium-permeable, Zn²⁺-sensitive channel in reconstituted lipid vesicles. *Biochemistry* 38:11189–11196.
- Lorenzo, A., Razzaboni, B., Weir, G.C., and Yankner, B.A. (1994). Pancreatic islet cell toxicity of amylin associated with type-2 diabetes mellitus. *Nature* 368:756–760.
- Mattson M.P., Barger S.W., Cheng B., Lieberburg I., Smith-Swintosky V.L., and Rydel R.E. (1993). beta-Amyloid precursor protein metabolites and loss of neuronal Ca²⁺ homeostasis in Alzheimer's disease. *Trends Neurosci.* 16:409–414.
- McKeith, I., Mintzer, J., Aarsland, D., Burn, D., Chiu, H., Cohen-Mansfield, J., Dickson, D., Dubois, B., Duda, J.E., Feldman, H., Gauthier, S., Halliday, G., Lawlor, B., Lippa, C., Lopez, O.L., Carlos Machado, J., O'Brien, J., Playfer, J., and Reid, W. (2004). Dementia with Lewy bodies. *Lancet Neurol.* 3:19–28.
- Merlini G., and Bellotti V. (2003). Molecular mechanisms of amyloidosis. *N. Engl. J. Med.* 349:583–596.
- Micelli, S., Meleleo, D., Picciarelli, V., and Gallucci, E. (2004). Effect of sterols on beta-amyloid peptide (AbetaP 1–40) channel formation and their properties in planar lipid membranes. *Biophys. J.* 86:2231–2237.
- Mirzabekov, T., Lin, M.C., Yuan, W.L., Marshall, P.J., Carman, M., Tomaselli, K., Lieberburg, I., and Kagan, B.L. (1994). Channel formation in planar lipid bilayers by a neurotoxic fragment of the beta-amyloid peptide. *Biochem. Biophys. Res. Commun.* 202:1142–1148.
- Mirzabekov, T.A., Lin, M.C., and Kagan, B.L. (1996). Pore formation by the cytotoxic islet amyloid peptide amylin. *J. Biol. Chem.* 271:1988–1992.
- Moechars, D., Lorent, K., and Van Leuven, F. (1999). Premature death in transgenic mice that overexpress a mutant amyloid precursor protein is preceded by severe neurodegeneration and apoptosis. *Neuroscience* 91:819–830.
- Monoi, H., Futaki, S., Kugimiya, S., Minakata, H., and Yoshihara, K. (2000). Poly-L-glutamine forms cation channels: relevance to the pathogenesis of the polyglutamine diseases [see comments]. *Biophys. J.* 78:2892–2899.
- Ng, A.W., Wasan, K.M., and Lopez-Berestein, G. (2003). Development of liposomal polyene antibiotics: an historical perspective. *J. Pharm. Pharm. Sci.* 6:67–83.
- Pan, K.M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R.J., and Cohen, F.E. (1993). Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc. Natl. Acad. Sci. USA* 90:10962–10966.
- Panov, A.V., Gutekunst, C.A., Leavitt, B.R., Hayden, M.R., Burke, J.R., Strittmatter, W.J., and Greenamyre, J.T. (2002). Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. *Nat. Neurosci.* 5:731–736.
- Parks, J.K., Smith, T.S., Trimmer, P.A., Bennett, J.P., Jr., and Parker, W.D., Jr. (2001). Neurotoxic Abeta peptides increase oxidative stress in vivo through NMDA-receptor and nitric-oxide-synthase mechanisms, and inhibit complex IV activity and induce a mitochondrial permeability transition in vitro. *J. Neurochem.* 76:1050–1056.

- Ramachandran, R., Tweten, R.K., and Johnson, A.E. (2004). Membrane-dependent conformational changes initiate cholesterol-dependent cytolysin oligomerization and intersubunit beta-strand alignment. *Nat. Struct. Mol. Biol.* Epub 2004 July 04.
- Reed, J.C. (2000). Mechanisms of apoptosis. *Am. J. Pathol.* 157:1415–1430.
- Relini, A., Torrassa, S., Rolandi, R., Gliozzi, A., Rosano, C., Canale, C., Bolognesi, M., Plakoutsi, G., Bucciantini, M., Chiti, F., and Stefani, M. (2004). Monitoring the process of HypF fibrillization and liposome permeabilization by protofibrils. *J. Mol. Biol.* 338:943–957.
- Rodriguez, C.M., Sola, S., Silva, R., and Brites, D. (2000). Bilirubin and amyloid-beta peptide induce cytochrome *c* release through mitochondrial membrane permeabilization. *Mol. Med.* 6(11):936–946.
- Ryou, C., Legname, G., Peretz, D., Craig, J.C., Baldwin, M.A., and Prusiner, S.B. (2003). Differential inhibition of prion propagation by enantiomers of quinacrine. *Lab. Invest.* 83(6):837–843.
- Sandberg, M.K., Wallen, P., Wikstrom, M.A., and Kristensson, K. (2004). Scrapie-infected GT1–1 cells show impaired function of voltage-gated N-type calcium channels (Ca(v) 2.2) which is ameliorated by quinacrine treatment. *Neurobiol. Dis.* 15:143–151.
- Sanderson, K.L., Butler, L., and Ingram, V.M. (1997). Aggregates of a beta-amyloid peptide are required to induce calcium currents in neuron-like human teratocarcinoma cells: relation to Alzheimer's disease. *Brain Res.* 744:7–14.
- Schein, S.J., Kagan, B.L., and Finkelstein, A. (1978). Colicin K acts by forming voltage-dependent channels in phospholipid bilayer membranes. *Nature* 276:159–163.
- Selkoe, D.J. (2002). Alzheimer's disease is a synaptic failure. *Science* 298:789–791.
- Simmons, M.A., and Schneider, C.R. (1993). Amyloid beta peptides act directly on single neurons. *Neurosci. Lett.* 150(2):133–136.
- Sipe, J.D. (2000). Serum amyloid A: from fibril to function. Current status. *Amyloid* 7:10–12.
- Sipe, J.D., and Cohen, A.S. (2000). Review: history of the amyloid fibril. *J. Struct. Biol.* 130:88–98.
- Sokolov, Y., Mirzabekov T., Martin, D.W., Lehrer, R.I., and Kagan, B.L. (1999). Membrane channel formation by antimicrobial protegrins. *Biochim. Biophys. Acta* 1420:23–29.
- Song, L., Hobaugh, M.R., Shustak, C., Cheley, S., Bayley, H., and Gouaux, J.E. (1996). Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. *Science* 274:1859–1866.
- Soto, C. (2003). Unfolding the role of protein misfolding in neurodegenerative diseases. *Nat. Rev. Neurosci.* 4(1): 49–60.
- Stipani, V., Galluci, E., Micelli, S., Picciarelli, V., and Benz, R. (2001). Channel formation by salmon and human calcitonin in black lipid membranes. *Biophys. J.* 81(6):3332–3338.
- Volles, M.J., and Lansbury, P.T., Jr. (2002). Vesicle permeabilization by protofibrillar alpha-synuclein is sensitive to Parkinson's disease-linked mutations and occurs by a pore-like mechanism. *Biochemistry* 41:4595–4602.
- Walsh, D.M., Klyubin, I., Fadeeva, J.V., Cullen, W.K., Anwyl, R., Wolfe, M.S., Rowan, M.J., and Selkoe, D. J. (2002). Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* 416:535–539.
- Westermarck, P., and Wilander, E. (1978). The influence of amyloid deposits on the islet volume in maturity onset diabetes mellitus. *Diabetologia* 15:417–421.
- Zhu, Y.J., Lin H., and Lal, R. (2000). Fresh and nonfibrillar amyloid beta protein(1–40) induces rapid cellular degeneration in aged human fibroblasts: evidence for AbetaP-channel-mediated cellular toxicity. *FASEB J.* 14:1244–1254.

Visualization of Protein Deposits *In Vivo*

Congo Red Staining of Amyloid: Improvements and Practical Guide for a More Precise Diagnosis of Amyloid and the Different Amyloidoses

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1. Abstract

Congo red (CR) is the most popular dye used as a probe for diagnosing amyloidosis, a very heterogeneous group of diseases with more than 23 chemically different amyloid syndromes of men and animals, leading to more than 400 different individual diseases. Congo red binding increases the natural anisotropy of amyloid, indicating that the elongated and planar CR molecules are aligned parallel to the axis of the amyloid α fibrils and to each other, thereby revealing a structure of amyloid. This structure was established to represent fibrils of similar dimensions, although the amyloid fibrils can be composed of many unrelated proteins. This CR-induced (positive) anisotropy displaying a green color is the hallmark of all amyloids, and is therefore used in the diagnosis of amyloidosis. The specificity of this criterion, however, is based on very stringent conditions of staining and evaluation. This review will focus on the understanding of the CR staining procedure, its mechanism and, in particular, on its recent practical improvements by increasing the sensitivity of the CR procedure, so that minute and the earliest amyloid deposits in the course of amyloidosis can now be reliably detected in patients. This enables a very early diagnosis in the course of the disease before irreversible organ damage might have occurred, and widens the options for a successful therapy. In addition, the central role of the CR diagnostic procedure and evasion of common pitfalls in arriving at a pathogenetically exact classification must only be based on the chemical nature of the amyloid deposits and not on the soluble precursors of the many different amyloidoses will be highlighted. The proposed bench-to-bedside algorithm will enable the physician to arrive at the exact diagnosis for therapeutic considerations. Finally, some possible future applications of CR and analogues will be presented.

2. Amyloidosis

The *amyloidoses* are members of a disease group caused by reduced protein catabolism, resulting in pathogenic protein depositions, called amyloid, in various organs. Therefore, these diseases have also been referred to as *protein storage diseases* (protein thesauroses). In addition, because an alternative protein folding leading to an enzyme-resistant conformation is crucial for the polymerization of these proteins, these disorders have also been called *conformational* and *protein folding dis-*

orders (Glenner, 1980; Bellotti and Merlini, 1996; Carrell, 1997; Sunde and Blake, 1998; Dobson, 2003; Merlini and Bellotti, 2003).

Amyloid is defined by the specific binding of CR (Bennhold, 1922a, 1922b, 1923), the increased optical anisotropy after CR binding (Divry and Florking, 1927; Romhányi, 1943; Missmahl, 1950), the characteristic green birefringence (Ladewig, 1945; Missmahl and Hartwig, 1953; Dietzel and Pfeleiderer, 1959; Wolman and Bubis, 1965; Romhányi, 1971), the fibrillar nature of straight, rigid and unbranched fibrils with a mean diameter of 10 nm (Cohen and Calkins, 1959; Spiro, 1959; Caesar *et al.*, 1960), the β -pleated sheet structure by X-ray diffraction (Eanes and Glenner, 1968; Bonar *et al.*, 1969; Shmueli *et al.*, 1969; Glenner *et al.*, 1974; Serpell *et al.*, 1999), by infrared spectroscopy (Termine *et al.*, 1972; Glenner *et al.*, 1974; Caughey *et al.*, 1991; Landsbury, 1992), and circular dichroism (Townsend *et al.*, 1966; McCubbin *et al.*, 1988). The properties of *ex vivo* amyloid extend also to amyloid-like fibrils formed *in vitro* (Glenner *et al.*, 1971a, 1974; Linke *et al.*, 1973).

Amyloidoses include a large number of different diseases that are tinctorially recognized and individually characterized by the chemistry of the deposited proteins, because the various associated symptoms and syndromes reflecting the different anatomical sites of amyloid depositions are less distinctive than the chemical nature of amyloid (see Section 7). This amyloid and the change in protein conformation leading to amyloid represent the *ens morbi* (the essential cause of the disease) for this entire group of illnesses, which today includes by far more than 400 individual diseases that can be classified into at least 23 different syndromes (Westermarck *et al.*, 2002; Buxbaum, 2004; Merlini and Westermarck, 2004).

Amyloidoses not only develop sporadically, but may demonstrate familial patterns of autosomal-dominant or recessive inheritance as well (Glenner, 1980; Benson, 1995, 2003; Buxbaum and Tagoe, 2000a). In general, the sporadic cases are more likely to appear at a more advanced age, while hereditary cases are generally observed decades earlier. One can distinguish between a local, organ-limited, or generalized deposition of amyloid, whereby the more generalized deposits are seen to demonstrate especially pathogenic characteristics, a feature that is reflected in their inexorably progressive character and their frequently fatal outcome (Glenner, 1980; Kyle and Gertz, 1995; Falk and Skinner, 2000; Merlini and Westermarck, 2004). For some of these illnesses, these processes leading to the progression of amyloidosis can today be prevented (Zemer *et al.*, 1986; Ben-Chetrit and Levy, 1991), reduced, or partially or completely arrested, with the chance of resolution of amyloid deposits through the use of specific therapies that have improved the prognosis and the well-being of the patient (Holmgren *et al.*, 1993; Ericzon *et al.*, 1995; Suhr *et al.*, 1995; Falk and Skinner, 2000; Lachmann *et al.*, 2003; Dispensieri *et al.*, 2004; Gono *et al.*, 2004; Herlenius *et al.*, 2004; Perz *et al.*, 2004; Seldin *et al.*, 2004; Skinner *et al.*, 2004). Because some of the different amyloidosis can now be treated, an accurate pathogenically correct diagnosis is mandatory to identify the individual diseases and distinguish them from all the other unrelated diseases.

Earlier classifications of these very multifarious and in some cases very rare illnesses were based on criteria other than chemical characteristics. Therefore, they did not lead to a consistent nosology in all aspects of these diseases (Reiman *et al.*, 1935; Heller *et al.*, 1964; Isobe and Osserman, 1974). Thus, therapeutic strategies and applications were rather limited. Pathogenetically meaningful therapies of the different amyloidoses only became known when diseases could be grouped together based on a similar pathogenesis, meaning disorders that are caused by the same or a very similar protein of origin as pioneered by Glenner *et al.* (1976) and Benditt and Erikson (1971). Today, more than 23 amyloid proteins have been detected (Westermarck *et al.*, 2002; Buxbaum, 2004), and the pathogenic ones of these need to be differentiated in patients. The application of this classification based on the chemical nature of amyloids for a precise diagnosis in patients is still hindered by the uncharacteristic early symptoms of the beginning amyloidosis. The *first most decisive factor* for an early diagnosis, therefore, is the *suspicion of the clinician*, because he/she will order the taking of a

biopsy, which subsequently will be examined for the presence of amyloid. The *second most decisive factor* will be the application of the *CR procedure*, which comprises the CR staining technique and the evaluation. Because the CR procedure is rather insensitive (Cooper, 1974; Hawkings, 1994; Linke *et al.*, 1995), and early amyloid deposits in biopsies can all too frequently escape detection by the usual evaluation procedures, high-sensitivity methods have been applied, and have thereby advanced the diagnosis of early amyloidosis (Linke *et al.*, 1995; Michels and Linke, 1998; Linke, 2000).

3. Identification of Amyloid Using Dyes

3.1. Introduction

This section will cite some historical and more recent techniques that have been applied in the effort to identify and analyze the amyloid deposits in different tissues and organs. This also includes the unveiling of the composition of amyloid, and some of the reasons that lead to the profound conformational changes that take place during the amyloidogenic transformation (Sunde and Blake, 1998; Dobson, 2003). Staining methods have been and continue to be instrumental in diagnosing the vast number of very different amyloid diseases known today and others yet to be discovered. Therefore, the *CR procedure* as an initial diagnostic method, and its recent improvements for identifying as well as further classifying the amyloidoses are discussed here in particular. Some reviews will be cited concerning very early concepts of amyloid (Puchtler and Sweat, 1966), and the use of various stains, in particular CR (Puchtler *et al.*, 1985; Cooper, 1981; Glenner, 1981; Westermark *et al.*, 1999).

3.2. Staining of Amyloid Before 1922

From the 17th century on (Kyle, 2001) the gross pathologic inspection of organs *in tabula* (macroscopy) revealed pale, enlarged, and indurated organs that could also be brittle. At that time this diagnosis was performed post mortem and a clinical picture had not yet been recognized, although this condition was occasionally associated with suppurative consumptive diseases. The first staining method was that of Virchow (1854) using Lugol's solution (alcoholic iodine), which he applied onto unfixed organs resulting in a mahogany-brown color that turned blue upon the addition of diluted sulfuric acid and demonstrated a behavior that was similar to cellulose. Despite this finding, Virchow did not refer to this pathologic condition as being "cellulose-like," but instead applied the botanical term "amyloid," meaning opposite to what he found "starch-like" (Puchtler *et al.*, 1985). However, Virchow's method did not stain amyloids consistently, and other dyes, such as the aniline dyes (methyl violet, crystal violet, and toluidine blue) have also been employed. Aniline dyes stain amyloid a reddish-violet in color (Carnes and Foker, 1956; Dietzel and Pfeleiderer, 1959; Cooper, 1969, 1974; Glenner, 1981). However, the aniline dyes also displayed inconsistent results (Cooper, 1969).

Although these early methods to diagnose amyloid in tissue sections are not used any further as routine tools today, they have added important clues to the structure based on results of the various staining techniques. Missmahl (1950) examining human *ex vivo* amyloid after a passage through the murine intestinal tract showed that the CR staining and the green birefringence remained untouched, while the staining with Lugol's solution and the metachromasia with methyl violet had disappeared, and that amyloid was enzyme resistant. The same result was found after *in vitro* pepsin digestion. The author concluded that only the Congo red staining with the green birefringence is specific for amyloid, proving the assumption of Ladewig (1945), and using only this criterion for diagnosing amyloid since. Using aniline dyes, Pras and Schubert (1969) were also able to distinguish between the disease-specific amyloid fibril and the unspecific "acid mucopolysaccharides." This dis-

tion was confirmed (Pras *et al.*, 1971) after the amyloid fibril could be isolated in a pure form using differential centrifugation (Pras *et al.*, 1968). These data demonstrated that some of the stains used for the detection of the amyloid deposit were specific for amyloid while others were specific for the associated materials. These findings were confirmed and extended upon by Cooper (1969, 1974, 1981). Therefore, it seems that substances inducing the iodine reaction, which gave the amyloid its name, may not be part of the fibril. In addition, Dietzel and Pfeiderer (1959) and Cooper (1976) showed that also many different mostly elongated dye molecules were aligned along the amyloid fibril and showed colored birefringence; these included toluidine blue, Sirius red, and even Eosine, confirming in part reports of others (Wolman, 1971; Puchtler *et al.*, 1985; DeLellis *et al.*, 1968). These and other data instigated examination for the substances consistently associated with the amyloid fibril such as the “acid mucopolysaccharides,” the amyloid-P component, and ApoE (Snow *et al.*, 1987; Strittmacher and Roses, 1996; Kisilevsky and Fraser, 1997; Pepys *et al.*, 1997; Kisilevsky, 2000), which has now become a major area in amyloid research.

3.3. CR as a Diagnostic Tool (Since 1922)

Measuring the blood volume in patients utilizing the degree of dilution of a standardized amount of CR, Bennhold (1922a, 1923) noted an unexpected rapid loss of the CR from the circulation in one of his patients. Very surprisingly, this patient had amyloidosis, as was later diagnosed at autopsy, and the entire amyloid mass was selectively stained with CR. This loss of CR from the circulation was later used to diagnose amyloidosis *in vivo*, but was discontinued when adverse effects were noted. In addition, this method appeared to be rather insensitive in early amyloidosis (Bennhold, 1923). In addition, CR binding to amyloid was also utilized to stain amyloid in tissue sections (Bennhold, 1922b), but this CR staining technique was frequently found to be unspecific. Therefore, attempts have been made by various groups to achieve specificity.

Divry and Florkins (1927) introduced polarization microscopy to the analysis of amyloid. They recognized the natural uncolored birefringence of amyloid and a strong increase in the anisotropy through the binding of CR. This increase was interpreted as a parallel alignment of CR in an ordered fashion by amyloid demonstrating “la structure cristalline de l’amyloïde” that was specific for amyloid (Divry and Florkins, 1927). Therefore, amyloid did not appear anymore to be amorphous but to have a structure that needed to be identified. Both findings were rediscovered by Romhányi (1943, 1949, 1959) and Ladewig (1945). However, the green birefringence was first described by Ladewig (1945) and Missmahl and Hartwig (1953). The appearance of the green anisotropy that characterizes amyloid in tissue sections rather than the CR binding continues to be the most decisive sign for the diagnosis of amyloid until this very day, although the CR staining techniques had only be refined during the period 1946–1962. This was achieved by Highman (1946), Puchtler *et al.* (1962), and Romhányi (1971). Although these methods varied, they virtually resulted in specific staining of amyloid for practical use, with the Puchtler’s staining method accepted as being the most specific.

Puchtler’s group performed the most thorough examination on the application of CR binding to the amyloid by applying the principles of cotton dyeing including CR analogues and many other cotton dyes for diagnosing amyloid (Puchtler *et al.*, 1962, 1964, 1985; Sweat and Puchtler, 1965). This group also described the sensitivity increase using fluorescence, that is, the CR bound to amyloid as a fluorochrome (Puchtler and Sweat, 1965) and an optical brightener for cellulose (Waldrop *et al.*, 1973). In addition, Mesitol WLS has been shown to improve the CR staining of very old sections (Meloan and Puchtler, 1978). The Puchtler method resulted in a dramatic increase in specificity compared to Bennhold’s method. Therefore, Puchtler’s “alcoholic alkaline CR staining method” has been accepted as the standard technique for identification of amyloid both *ex vivo* and *in vitro*. Our own experience with Puchtler’s method over the course of decades has shown *reliability* based on

the lack of falsely positive and falsely negative results when applied and evaluated properly, consistent with the experience of other centers involved in diagnosing amyloidosis (Cohen, 1967; Glenner, 1980, 1981; Westermark *et al.*, 1999).

3.4. Some Current Staining Protocols Using CR

Because practical information will promote the understanding of what is needed for rendering the CR procedure as being specific, the *core* of a few CR staining methods will be presented briefly.

The original method of Bennhold (1922b) applies 1% CR in distilled water onto hydrated tissue sections (up to 20 minutes) followed by dipping into an aqueous solution of saturated lithium carbonate for up to 15 seconds, followed by 80% ethanol for differentiation. The method of Highman (1946) stains hydrated sections with 0.5% CR or Congo corinth G in 50% alcohol for 1–5 minutes. After a wash in water the sections are differentiated in 80% ethanol containing 0.2% potassium hydroxide. The method of Romhányi (1971) applies CR at 0.1% CR in distilled water for 10 minutes onto hydrated tissue sections followed by washing out the unbound CR for 30 minutes in running tap water before embedding the section in gum arabic. The CR staining method of Puchtler *et al.* (1962) is more time consuming, but has the advantage of a proven specificity as demonstrated by evaluations made worldwide and decades of experience. Hydrated tissue sections are first exposed to solution Ia (80% ethanol in distilled water and saturated NaCl; with 1 ml of 1% aqueous NaOH added to 100 ml just before use) for 20 minutes followed by solution IIa (solution I with saturated CR); also, here, the alkaline (see above) is added just before use. Dehydrate rapidly in three changes of absolute ethanol, Xylol, and Permout. The solutions (Ia and IIa/b) need to be freshly prepared approximately every 2 months when they are kept in stained light impermeable glass bottles or in the dark, because CR is a light-sensitive stain (Puchtler *et al.*, 1962).

An *improved CR staining method*, which is based on the principles of the procedure of Puchtler *et al.* (1962), has been developed and applied successfully in my laboratory. This method uses a higher concentration of CR in the second solution (IIb), which thereby reduces the time of staining to only a few minutes. As illustrated in Figure 11.1-1, the CR concentration of 0.3 mg/ml in solution IIa was increased to 1.1 mg/ml in solution IIb.

This improved method commences with solution Ia with alkaline solution (see Puchtler) being applied onto hydrated tissue sections for 10 minutes, followed by solution IIb for 1–5 minutes according to the requirements, because the different amyloids bind different amounts of CR (Westermark *et al.*, 1999; Linke, 2000). Application of solution IIb for only seconds is described in Section 8.4. Solution IIb is prepared 1 day before use by adding to 10 ml of saturated CR (52 mg/ml) 80% ethanol/saturated NaCl in distilled water up to a volume of 100 ml. The final concentration of ethanol is 72%. Alkaline solution is added just before use (see above).

3.5. Staining of Amyloid-Like Fibrils with CR

When protein precipitates which are formed *in vitro* from various peptides or polypeptides are being examined for the presence of amyloid characteristics, the staining method used is not always described in detail in the literature. The reason could be that the Puchtler's method cannot always be applied due to the fact that artificial amyloid-like fibrils are less stable as compared to *ex vivo* amyloid fibril preparations. Thus, the former may be dissolved due to the alkaline conditions. When *ex vivo* amyloid fibril preparations are examined for their content of amyloid, they can be dried onto glass slides in a drop of 5–10% serum and stained with CR using Puchtler's staining technique, in the same manner as the staining of fixed tissue sections.

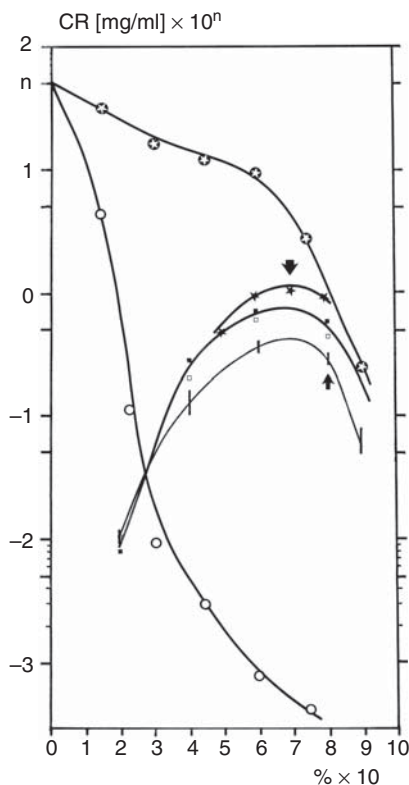


Figure 11.1-1. Increasing the concentration of CR for a quick CR staining method. Maximal concentration of CR as a function of % of ethanol (white stars) or % saturated NaCl (open rings). Fat arrow points to maximal CR concentration at 1.1 mg/ml (conditions: 72% ethanol and 82% saturated salt (black stars, CR added, saturated in distilled water at 52 mg/ml). Small arrow points to maximal concentration of CR (0.3 mg/ml); conditions of Puchtler *et al.* (1962), that is, 80% ethanol and saturated NaCl. Small squares indicate maximal CR concentration in two experiments (open and filled square) in saturated NaCl with varying concentrations of ethanol with CR added as saturated solution in distilled water. Bars indicate standard deviation of three experiments with CR added as solid. Ordinate: log CR concentration; All other curves: % ethanol in distilled water

In contrast, the suspension of amyloid-like fibrils formed *in vitro* should always be incorporated into a small drop of 5–10% human serum (or another proteinaceous carrier) and air dried on glass slides. The dried section will then be fixed in either 4% buffered formaldehyde (= 10% formalin) or in 2% buffered glutaraldehyde for 1 hour at room temperature when they tend to be dissolved. After washing in tap water and blocking with small alkaline proteins or small amines the fixed amyloid will be air dried and finally dried down in an oven at 55°C before being stained according to Puchtler *et al.* (1962).

3.6. CR as a Fluorochrome

The use of CR as a fluorochrome (CRF) utilizing its increased sensitivity was first reported by Fahr (1944) as cited by Missmahl (1950). Cohen *et al.* (1959) also reported CRF and its increased sensitivity but also its unspecificity. However, when CRF was reevaluated using their improved staining method by Puchtler *et al.* (1962), its high sensitivity and its specificity for detecting amyloid was emphasized. Use of techniques for increasing the sensitivity of the CR procedure, however, became

mandatory for routine use when, as demonstrated using immunohistochemistry, early stages of amyloidoses were documented to have been missed in 90% of early biopsies (Linke *et al.*, 1995), due to the relative insensitivity of the common CR procedure. In addition, using CRF, an additional method for increasing the sensitivity of the CR procedure showed that the incidence of amyloid detected was seen to be doubled in biopsies with sparse amyloid deposits (Linke, 2000). These data also show that the suspicion by the clinician, which is marked by the ordering of a biopsy, was far more sensitive than the common CR procedure (see Section 6.8). This relative insensitivity of the common CR procedure (which is still the routine procedure in most institutes of pathology) also explains why a negative bioptic amyloid diagnosis performed with CR alone always remains inconclusive (see Section 6.12).

3.7. Thioflavin

Increased sensitivity for detecting amyloid has been described using thioflavin T and S, as introduced by Vassar and Cullings (1959). These fluorochromes, being basic dyes and binding to acidic structures in tissue sections, have been widely applied in amyloid research (Stiller and Katenkamp, 1970). They are frequently used for screening purposes on tissue sections and amyloid-like fibrils formed *in vitro*, because of their bright yellow-green fluorescence that can be easily detected and experimentally traced. These dyes, however, have been found to also bind to other structures than amyloid. Because they are considered to be unspecific for amyloids, the fluorescent reactions should be controlled in every case by more specific methods such as CR (Stiller and Katenkamp, 1970; Stiller *et al.*, 1972; Cooper, 1976; Glenner, 1981; Puchtler *et al.*, 1985; Westermark *et al.*, 1999). Because the conventional thioflavine T staining was found to be inconsistent, it can be replaced by the optical brightener for cellulose, Phorwhite BBU, by Waldrop *et al.* (1973).

3.8. Other Dyes and CR Analogues

Diezel and Pfeleiderer (1959) applied a host of different dyes and other compounds to amyloids and showed that many dyes can be aligned along the amyloid fibril axis, and some of them display colored anisotropy showing the respective complementary color under crossed Nicols. So, yellow-green anisotropy is seen with such red colors as CR, Congo corinth, Sirius red, Thiazin red, and Eosine, while binding to amyloid of such blue or violet colors as Evans Blue and Toluidine blue induce orange-red anisotropy in polarized light (Dietzel and Pfeleiderer, 1959; Wolman, 1971).

Puchtler *et al.* (1962) and Sweat and Puchtler (1965) not only reported the most successful method for staining amyloid specifically using CR, but also introduced CR analogous and other cotton dyes such as Sirius red and F3BA, which they reported to be as specific, and in particular, very sensitive for diagnosing amyloids. Many of these dyes have not yet been employed on a larger scale on the many chemically different amyloid types that we know today (Westermark *et al.*, 2002; Buxbaum, 2004). Some of the many CR analogues that can bind to amyloids have been used, however. One such analogue, chrysamine G (see Figure 11.1-2), which binds to amyloids *in vitro* (Dezutter *et al.*, 2001), has been used successfully in diagnosing amyloids in whole-body radioactive imaging in chickens with AA-amyloidosis. This tracer was able to identify the characteristic joint and liver amyloid while the amyloid-P component (Hawkins *et al.*, 1990; Hawkins *et al.*, 1995), used for comparison, did not mark the articular amyloid (Dezutter, 2001). Other results will be mentioned in Section 11.

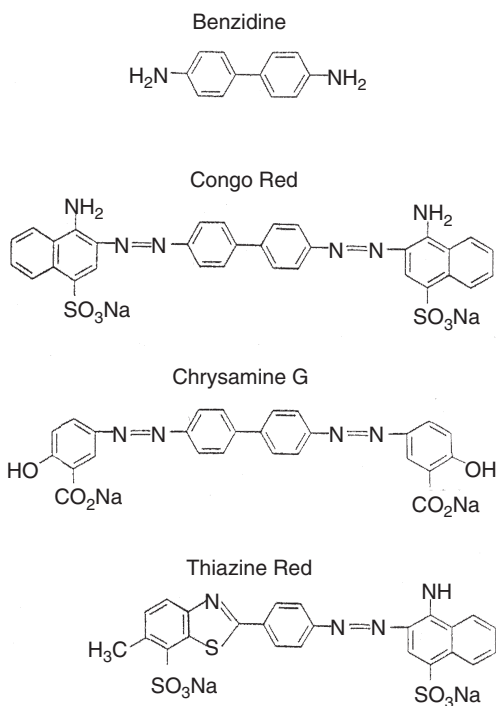


Figure 11.1-2. Chemical structure of CR and analogues. The diazids CR and the CR analogue chrysamine G, and their hydrophobic core benzidine are shown, as well as Thiamin red, a monoazid compound (see Section 4.1). All exhibit colored anisotropy after binding to amyloid (see Sections 4.2–4.4)

4. The Chemical Structure of CR and Some Properties

4.1. History and Chemistry

Congo Red was discovered in 1883 by Paul Böttiger, who was working as a dye chemist for the Friedrich Bayer Company in Ebersfeld, Germany, and named “Congo” in 1885 for marketing reasons, as reviewed in all historical details by Steensma (2001). Congo Red was used as a cotton dye. It was the first “direct” dye that could be used without any pretreatment of the cotton. Congo red is an azo dye, which is derived from bisdiazotized benzidine substituted with two molecules of naphthionic acid. The synthesized blue stain turns red upon exposure to NaCl. Congo Red is chemically (3,3'-(4,4'-biphenyldiylbisazo)bis(4-amino-1-naphthalinsulfonic acid) disodium salt) with a molecular weight of 697 Da. The disadvantages of CR are its light sensitivity and its derivation from benzidine, a known carcinogen (Falbe and Regitz, 1990). The industrial product CR, which was used for diagnostic purposes earlier, consisted of more than 20 different fractions as separated on thin-layer chromatography, but not all of the bands were red and not all red bands stained amyloids equally well. Which of the different constituents had adverse effects, as noticed *in vivo* by Bennhold, is unknown. These impure products are obsolete today. As shown in Figure 11.1-2, CR is a bisulfonated charged molecule. It has an multiring structure, which is in resonance, thus leading to a flat and

elongated configuration of the molecule. However, the central bond between the two symmetrical parts of CR shows free rotation (Skowronek *et al.*, 2000).

4.2. Characteristics of CR Crystals

Congo red crystallizes easily, and some of the properties of these crystals have been described (Wolman and Bubis, 1965). Because their findings are crucial for understanding the CR binding to amyloid and the associated optical phenomena, some will be repeated in some detail. When an aqueous CR solution in 60% alcohol is allowed to evaporate slowly, multiple, deep red, hair-like crystals are formed, as shown in bright light (**Figure 11.1-3a; see color insert**). Between crossed Nicols, however, the same crystals appear in different colors, dependent on their diameter (**Figure 11.1-3b; see color insert**), with the thinnest being green. With increasing thickness, these crystals subsequently turn yellow-green, yellow, orange, and red. This colored anisotropy (birefringence) is characteristic of CR crystals, and the color change has been explained in detail (Wolman and Bubis, 1966). They proposed that the green birefringence is the result of a half wavelength retardation of the red light by the CR-stained amyloid (standard thickness of tissue section) compared to the white light wiping out the red fraction of the white light yielding the respective complementary color yellow-green. However, when viewed under green excitation, all the hair-like CR crystals fluoresce in bright red, regardless of their thickness (**Figure 11.1-3c; see color insert**). Most importantly, CR that is accommodated in parallel along amyloid fibrils reveals the same characteristics as CR hair-like crystals. To denote this similarity, one sometimes speaks of CR being aligned along the amyloid fibril in the form of a “*para-crystal*.”

In contrast, when CR is evaporated from a concentrated aqueous CR solution, a thick, deep red “cake” is formed that does not show any birefringence, although it also represents CR crystals. When this cake is scratched, CR crystals are oriented along the direction of the scratch and show anisotropy (**Figure 11.1-3e and f; see color insert**). Interestingly, in polarized light they display the same colored birefringence known from CR crystals that are specific for amyloid of the respective thickness (**Figure 11.1-3b; see color insert**). In addition, the phenomenon of dichroism, which is also characteristic of amyloid after CR staining (Romhányi, 1949), and which is the precondition of the green birefringence (Wolman and Bubis, 1965; Wolman, 1971), could also be shown on the scratched (meaning aligned) CR crystals.

4.3. Concerning the Value of the Green Polarization Color

The “green polarization color” represents a restricted view considering the fact that amyloid displays various polarization colors dependent on the thickness of the sections based on results by different groups (Diezel and Pfeleiderer, 1959; Wolman and Bubis, 1965; Cooper, 1974). Thus, the more precise view would be that amyloid is characterized by the “colored birefringence” (Cooper, 1981). As can be seen in **Figure 11.1-3b; see color insert**, the polarization colors yellow, orange, and red are *all* as specific for amyloid as is the green color. Because the green color, which verifies the presence of amyloid, cannot be taken as the only color specific for amyloid in the strict sense, it represents only an “accidental color,” which is the result of what happened by chance when tissue sections were standardized to be of 4–8 μm thickness. Therefore, the green anisotropy as proof for the presence of amyloid is only valid when tissue sections are of a standard thickness. With this in mind, one can state that the green polarization color is the most specific criterion for amyloid (Ladewig, 1945; Romhányi, 1949, 1971; Wolman and Bubis, 1965; DeLellis *et al.*, 1968; Glenner *et al.*, 1974), which is by far more specific than the CR binding alone and accordingly the CRF.

4.4. Colored Anisotropy After Binding of CR

The anisotropy of amyloid arises by the parallel packing of CR along the axis of the amyloid similar to crystallization (Divry and Florkins, 1927; Romhányi, 1943; Dietzel and Pfeleiderer, 1959; Wolman and Bubis, 1965; DeLellis *et al.*, 1968; Glenner *et al.*, 1974). In addition, the different colors of the anisotropy reported by Dietzel and Pfeleiderer (1959), and Wolman and Bubis (1965) were examined on tissue sections thinner than 4 μm . Interestingly, there is not only a color change, but also the fading of the polarization color with decreasing thickness. The amyloid turns from whitish-green to bluish-white and finally to almost white as first reported by Wolman and Bubis (1965), who also reflected the unfavorable implications for diagnosing amyloid in thin tissue sections, which we can confirm. It is clear that all these colors are *specific* for amyloids, except for the whitish anisotropy, which is indistinguishable from the birefringence of collagen confirming the results of Wolman and Bubis (1965). This phenomenon has posed a problem, for example, in nephrological pathology, where approximately 1- μm sections are examined for glomerulopathies. We were consulted to examine such sections, which neither showed CR staining nor clear green birefringence. To circumvent this problem we first had to demonstrate CR binding by using the CRF (Linke, 2000). The verification of the CR binding then demanded the preparation of tissue sections having standard size for the demonstration of the colored birefringence after CR staining because CR binding alone cannot prove the presence of amyloid (Cooper, 1969, 1981, 1991; Glenner, 1981). CR binding could be verified in our hands by CRF to a thickness of approximately 0.1 μm . In addition, when tissue sections are too thin, the colored birefringence cannot be used to distinguish amyloid from nonamyloidotic protein deposits. Such nonamyloidotic deposits and the related diseases have been reported and reviewed (Gallo *et al.*, 1980; Picken *et al.*, 1989; Buxbaum, 1992; Casanova *et al.*, 1992; Buxbaum *et al.*, 2000; Walker and LeVine, 2000). Although lack of CRF proves the lack of amyloids, because all amyloid binds CR, the reverse is not true. As shown, amyloids can only be verified when appropriate tissue sections can be furnished and examined (see also Section 6).

4.5. Mechanism of CR Binding to Amyloids

How this parallel alignment of CR along the amyloid fibril axis occurs has been a matter of debate and extended examination. Puchtler *et al.* (1962, 1964) and Cooper (1969, 1974, 1981) have examined and summarized the data on the binding of CR. It is accepted that ionic factors, and to some degree hydrogen bonds, have been eliminated by the Puchtler staining solution of saturated salt, alkaline, and high percent ethanol. In addition, peptic digestion and intestinal passage did not eliminate the CR binding properties (Missmahl, 1950; Cooper, 1974), but exposure to alkaline or 6M guanidine, which disintegrates the fibrillar structure of amyloids, indicating the integrity of the β -pleated sheet fibril to be the precondition of the CR binding and the green birefringence, while Eosine and methyl violet continued to bind even after the CR did not bind anymore, indicating a different binding mechanism of CR and the two latter dyes (DeLellis *et al.*, 1968). The assumption that CR binds by its conformation as an elongated molecule probably via its hydrophobic center (Figure 11.1-2) through short-range forces and Van der Waal's forces to particular sites of the amyloid is assumed by Cooper (1981) and proposed for other molecules by Edwards and Woody (1979).

Because amyloid-like fibrils show the same binding characteristics as *ex vivo* amyloid fibrils, these CR binding groups have to be provided by the amyloid protein, and will represent repetitive binding motifs in strict order along the axis of the amyloid fibril, which may be reminiscent of similar structures on cotton. The binding of CR similar to cotton, which acts through hydroxyl groups, as proposed by Puchtler *et al.* (1962), has been disputed by Cooper (1981, 1991), who reported that

alterations of hydroxyl groups did not eliminate the green anisotropy. These findings indicate that the binding of CR to cotton may be different from the binding to amyloid. When this is true, the linear hydroxyl bonds along the fibril axis that are operative in the cotton dyeing by CR are not operative in the CR binding to the amyloid (Cooper, 1981, 1991). The very strong binding between the elongated multiring structure CR (see Figure 11.1-2) and the amyloid could be furnished by elongated furrows (Cooper, 1981, 1991) or end-edge groups (Cooper, 1991) along the amyloid fibril, which can be considered a linear crystal (Jerret and Landsbury, 1993). A multisite binding of a single CR molecule may increase its binding avidity. This increased strength of binding could also be provided by the finding that CR could bind to amyloid via CR multimers as first reported by Wälti (1945). Interestingly, Roterman *et al.* (2001) recently reported the binding of CR heptamers rather than monomers to amyloid-like fibrils. How these CR polymers are accommodated along the amyloid (sort of microcrystals?) is unknown.

Congo red consists of two symmetrical planar halves that show torsion of the central biphenyl bond (Skowronek *et al.*, 2000). During crystallization or binding to amyloid-like fibrils, this freedom is lost by ordered accommodation of CR, which is restricted to an elongated-only planar molecule (Miura *et al.*, 2002). Although a proven molecular model of the amyloid fibril is still not reported, alternative models are presented. One model favors a β -helical structure of native *ex vivo* amyloid fibrils that appear to represent a tubular structure with a hole inside a single fibril as reviewed by Wetzel (2002), rather than a tightly twisted ribbon of several single filaments (Glenner *et al.*, 1974) or protofibrils (Serpell *et al.*, 1999), which are reported from amyloid-like fibrils created *in vivo* or possibly formed during the extraction procedure, as reviewed by Kisilevsky and Frazer (1997).

5. Concerning the Specificity of CR

There are very few conferences on amyloid and amyloidosis without a discussant asking the question: "Is CR really specific?" and just as frequently the following discussion is not clarifying at all. As usual, both parties are correct, meaning that CR can bind to most proteins through hydrophobic and/or ionic bonds, and CR is therefore *not* specific for amyloid on its own. Because CR can also be aligned along structural proteins such as the collagens in physiologic solutions, it will sometimes yield the green birefringence even in the absence of amyloid, and stringent conditions are necessary to render the CR staining method specific for amyloid. To arrive at specificity with respect to amyloid detection, two different parts of the CR procedure need to be seriously addressed: (1) the very *stringent staining conditions* of Puchtler *et al.* (1962) using CR (or equivalent stains and staining protocols) on 4–8 μm -thick tissue sections, and (2) the *profound morphologic experience* of the evaluator. The latter is required for the microscopic evaluation of the stained tissue sections. He/she has to recognize the apple-green birefringence in polarized light following CR staining of standard tissue sections (Ladewig, 1945; Missmahl and Hartwig, 1953; Cohen, 1967; Romhány, 1971; Glenner *et al.*, 1974). However, not in all cases does the green (or colored)-birefringent material represent amyloid. Typically, amyloid is situated extracellularly in tissues and organs at such typical anatomical locations as vessel walls, along cell membranes, in particular basement membranes, as well as along different collagen and elastic fibers. Amyloid can also be detected with variable distributions and classified as local, organ-limited, or systemic (Glenner *et al.*, 1974; Glenner, 1980). It can also present as stellar bodies, as plaques or as amyloid nodes or tumors (amyloidomata). Some of the different amyloid syndromes display some clinical and morphological peculiarities that one gets to know with experience and that were the basis of former classifications of the amyloidoses (Lubarsch, 1929; Reiman *et al.*, 1935; Isobe and Osserman, 1974). On the other hand, one also has to recognize all inappropri-

ate staining results. Overstaining may also display green birefringence of nonamyloidotic structures. In addition, there are even materials displaying green birefringence after correct CR staining without representing amyloid, such as some keloids, cotton fibers, fungi (Cooper, 1969), cellulose, and other plant materials, chitin, while other constituents such as elastin show only CR binding but no green birefringence (Puchtler *et al.*, 1962; Cooper, 1969). Any kind of “detritus” can sometimes be found to display green birefringence beside or on top of the tissue sections. These materials are easily excluded by their microscopic appearance. Also, crosscontamination of amyloid-free sections with floating amyloid flakes detached from amyloid-containing sections stained in the same jar may occur, which could mainly pose a problem in tissue smears. Also, hemoglobin, as seen commonly in subcutaneous fat aspirations, can sometimes display a greenish tinge, which could be misinterpreted as an amyloid. The latter case can be differentiated from amyloid by CRF (see Section 6.9). Finally, amyloid is not always situated extracellularly and paired helical filaments, endocrine amyloids, and Russel body-like inclusions of plasma cells can display some typical amyloid characteristics of amyloids. Whether some of these deposits are called amyloid is, however, still being discussed (Westermarck *et al.*, 2002). These examples may illustrate that CR is only specific for amyloid when handled appropriately.

6. Concerning the Practical Use of CR

In reviews and at meetings on diagnosing amyloidosis an unrecognized paradox is seen to be apparent. Although the impression is gained from reviews that the diagnosis of amyloid is no problem, provocative statements of some of our experts are uttered at meetings like: “I don’t care about CR” or “CR is totally unspecific,” as I witnessed during the discussion on the International Symposium on Amyloid and Amyloidosis in Tours (2004). This paradox can be solved by applying the CR procedure appropriately as shown above and as reviewed (Glenner *et al.*, 1974; Linke, 1987; Westermarck *et al.*, 1999; Buxbaum, 2004; Merlini and Westermarck, 2004). That the CR staining procedure is not trivial to perform was already mentioned by Waldrop *et al.* (1973). However, even when the above-mentioned *two parts of the CR procedure* are applied correctly, there could still remain some pitfalls that have to be addressed and, therefore, additional information is needed. Finally, it has also to be stressed that the electron microscopic demonstration of the presence of fibrils in tissue sections or produced *in vitro* having similar dimensions as amyloid (mean diameter of 10 nm) is ancillary for the diagnosis as is the radioactive imaging (Hawkins, 1994), and can never replace the CR procedure because (1) various fibrils resemble amyloid as some of the intermediate fibrils (Glenner *et al.*, 1974; Glenner, 1980; see Figure 11.1-5), and (2) the amyloid is defined by the binding of CR and its green anisotropy when the described stringent conditions are kept (see Section 3.3).

6.1. The Quality of Equipment

An appropriate microscope, especially equipped for polarization microscopy, is *the* prerequisite, including a well-centered light beam that is not deflected too often. Therefore, teaching microscopes with many additional microscopes attached should be checked with a CR-stained standard slide containing the amyloid to see whether they are useful. The amyloid after CR staining is examined between crossed Nicols using maximal light in a dimmed room (always used and recommended by Missmahl, personal communication). In addition, equipment for fluorescent microscopy is needed for high-sensitivity diagnosis of CR-stained tissue sections with filter sets for fluorescein-isothiocyanate with a broad barrier filter that allows the yellow-orange light to pass and/or respective filters for tetramethylrhodamine (Linke, 2000).

6.2. The Quality and Kind of the Biopsy

This point addresses the endoscopist. A freshly taken tissue biopsy should be fixed immediately (see Section 6.6). Rectal biopsies should contain the submucosa. Therefore, a biopsy containing only the mucosa may be useless when no amyloid can be detected, because the amyloid may only be present in the arterioles and arteries of the submucosa in some patients. Cryostat and fixed paraffin sections are feasible. Standard fixation with 4% buffered formaldehyde (= 10% buffered formalin) is appropriate. Even prolonged fixation in formalin is suitable for CR staining and even for immunohistochemistry and immunoelectron microscopy according to our experience (see Section 7.4). Biopsies were at first taken from gingiva (Selikoff and Robitzek, 1947) and rectum (Calkins and Cohen, 1960), but included later such other organs such as heart, kidney, intestine in general, liver, trachea, and paratracheal lung, sural nerve, muscle, various glands, skin, joint, and other tissues (Cohen, 1967; Glenner, 1980; Merlini and Westermark, 2004). Also, aspiration biopsies of subcutaneous fatty tissue have been successfully used for diagnosing amyloid (Westermark and Stenkvist, 1973; Westermark *et al.*, 1989; Arbustini *et al.*, 2002). Various amyloids were immunohistochemically classified from different biopsies as cited in Section 7.4.

6.3. The Size of the Biopsy and the Sampling Error

Any of the biopsies need a certain size, that should not be below 1 mm² if possible, because the very small sections may float off the glass slides during the staining procedures even when special slides prepared for immunohistochemistry are used (which is the standard today). In addition, we have shown that small biopsies are more prone to sampling error (see Sections 6.8 and 6.9), meaning that *not all* tissue sections cut from one block may contain amyloid. Thus, evaluation may be misleading when only a single section is examined. In case no amyloid is present, we examine 10–20 more tissue sections from the same paraffin block employing the high sensitivity CR procedure (Linke, 2000) for excluding the sampling error.

6.4. The Quality of Tissue Sections and Minute Amyloid Deposits

The quality of a section implies in particular its thickness and the tissue selected. Small amyloid deposits can be buried within thick tissue sections, as illustrated in Figure 11.1-4. In addition, thick sections pick up more counterstain (hemalum) than normal sections, which may conceal the CR stain and abolish the green birefringence when amyloid deposits are small (Westermark *et al.*, 1999). In addition, in normal tissue sections of 4–8 µm with amyloid deposits of below 2 µm, the amyloid may be shielded by normal tissue also (Figure 11.1-4), although with the disadvantage that the minute amyloid deposits picks up less CR (see Section 4.4). In this case, the amyloid is not stained red by CR (**Figure 11.1-3g; see color insert**) and the anisotropy may not display the colored birefringence (see Section 4.4). Biopsies of patients with very early and possibly small amyloid deposits are usually missed except when the high-sensitivity CR procedures are being applied (see Section 6.8, 6.9). This can be achieved using immunohistochemistry in humans and animals (Linke, 1987; Linke *et al.*, 1995; Schulz *et al.*, 1998) and by CRF (Puchtler and Sweat, 1965; Wolman and Bubis, 1965; Cooper, 1969; Linke, 2000).

6.5. The Quality of Staining

Every individual CR staining procedure should be controlled by the costaining of a tissue section that contains a high CR binding amyloid. For providing a consistently positive control and

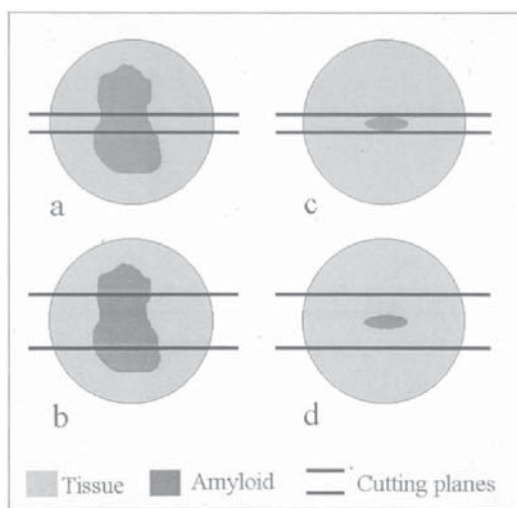


Figure 11.1-4. Diagnostic problems arising from minute amyloid deposits. Amyloid of normal size (a, b) is insensitive to the thickness of the section because both thin (a) and thick (b) sections expose amyloid equally well to the cutting plane. Very small amyloid deposits may benefit from thin sections (c) when the amyloid is hit and not missed, thereby resulting in a sampling error (see Section 6.3). In thick sections (d), amyloid may be concealed when covered with other tissue structures, especially when counterstained. Amyloid below $2\mu\text{m}$ in normal sections ($4\text{--}8\mu\text{m}$) may cause similar problems and have an additional disadvantage of len uptake of CR (see Section 6.4). These problems can be circumvented using CRF (see Section 6.9)

standard, this control section should always be cut from the same paraffin block, thus enabling the evaluator to verify the quality of the individual staining process. Because CR is light sensitive (Puchtler *et al.*, 1962; Falbe and Regitz, 1990), the performance of the CR solutions will be checked by this positive control as well. At the same time, altered results as a function of the thickness and overstaining can easily be identified (see Figure 11.1-4). Finally, using this kind of a control, nonamyloidotic protein deposits such as light-chain deposition disease or fibrillar glomerulopathies (Gallo *et al.*, 1980; Casanova *et al.*, 1992; Buxbaum *et al.*, 2000b; Walker and LeVine, 2000) can easily be recognized when it is clear from the positive control that the applied CR procedure was adequate.

We usually encounter problems when stained tissue sections have been sent to us for evaluation because they are, in almost all cases, inadequately stained. In this case we first examine a parallel section cut from the same tissue block stained by ourselves, and our evaluation will reveal the problem. In this way, we identified overstained necrotic tissues (tbc, viral necrosis) or scar tissue instead of the submitted amyloid diagnosis, or reversely, we identified the amyloid when similar diagnoses (“tumor necrosis, fatty tissue necrosis”) were presented for a second opinion.

6.6. Imbibition of Serum and Tissue Proteins

This point requires the attention of clinicians and surgeons. When a biopsy or a larger excision is sent to the pathologist for examination for the presence of amyloids, the time of fixation may be delayed for many reasons. This delay in time and the hours of autolysis may allow blood and tissue

Table 11.1-1. Resistance of antigenic determinants of amyloid toward fixation while unspecificity is lost at the same time: “Differential fixation” of amyloid

Processing			Immunohistochemical reactions		References
Fixation	Embedding	Microscope	Amyloid	Unspecificity	
No	No	LM	+++	+--+++	1
Formaldehyde	Paraffin	LM	+++	0—+	2
Formaldehyde	HM	LM, EM	+++	0—(+)	3
Formaldehyde and others	EP, NO, and others	EM	++ —+++	0—(+)	4

Relative resistance of antigenic determinants of amyloid toward fixation-induced denaturation while, at the same time, the background staining is virtual gone. This phenomenon called “differential fixation” leads to an increased specificity for immunohistochemical detection (see Figure 11.1-5, and Section 7.4) consistent with the selective preservation of amyloid fibril proteins in fixatives (see Section 7.5).
LM, light microscope; EM, electron microscope; HM, hydroxyethyl-methacrylate; EP, epon.
References: 1, unpublished; 2, Linke and Nathrath, 1980; 3, Donini *et al.*, 1984; 4, Linke *et al.*, 1989, Arbustini *et al.*, 1997.

proteins to enter the amyloid. Subsequent fixation will preserve these admixtures to the amyloid deposit, and may conceal the protein of origin, although this also happens to cryostat sections (Linke, 1985). This phenomenon was seen to occur more frequently in small native biopsies used for classification on cryostate sections, which resulted in multiple reactions in some cases. By use of a novel microextraction method for the classification of amyloid in 10–30-μg biopsies followed by immunochemical identification, a single amyloid protein was detected in every one of the 20 samples analyzed (Linke, 1985). To avoid imbibition and to utilize the fixation resistance of amyloid (see Table 11.1-1), we did not use cryostat sections anymore. Biopsies can either be rinsed free of serum proteins in physiologic salt solution and fixed thereafter or shaken immediately a few times after being dropped into the formalin solution.

6.7. Relative Insensitivity of the Conventional CR Staining Procedure

The insensitivity of the conventional CR procedure is an unfavorable feature as addressed by Cooper (1969), Hawkins *et al.* (1990) and many other authors can yield false negative results in patients with amyloidosis. The presence of amyloids in these sections could be diagnosed using procedures of increased sensitivity of the CR procedure, which will be discussed below (Sections 6.8 and 6.9)

6.8. Increased Sensitivity of the CR Procedure by Immunohistochemistry (CRIC)

To measure the sensitivity of the routine CR procedure versus the high-sensitivity version, a retrospective study on children with chronic juvenile inflammatory diseases was examined. Various biopsies were taken after clinicians suspected AA-amyloidosis, and these were evaluated by different institutes. The results on the earliest biopsies of children who later developed severe AA-amyloidosis were interesting in particular. Applying immunohistochemistry to CR-stained sections (CRIC), it was found that the early AA-amyloid present in biopsies was detected in only 10 (ten!)%. Using CRIC, the other 90% could easily be detected (Linke *et al.*, 1995), meaning that the historical results from the charts were burdened with a severe sampling error that was clarified immunohistochemi-

cally. Similar findings were reported for animals (Schulz *et al.*, 2001). The benefit of CRIC was (1) that the diagnosis of AA-amyloidosis has been achieved an average of 3 years earlier than with the use of the conventional CR procedure as retrieved from the charts, (2) earlier therapy by this time gain, and (3) avoiding further biopsies and various diagnostic measures (Michels and Linke, 1998).

6.9. Increased Sensitivity Using CRF

Usually, the green birefringence is described as more sensitive than CR alone (Missmahl and Hartwig, 1953; Wolman and Bubis, 1965; Romhányi, 1971), but it is prone to be missed (Cooper, 1969; Linke, 1985) due to the “polarisation shadow” (see Section 6.11). To overcome this problem CRF was employed, which displays an even higher sensitivity than CR and even CRIC. In addition, CRF illuminates the entire amyloid deposits in a tissue section at the same time (see Sections 6.8, 6.9, and **Figure 11.1-3; see color insert**). CRF has been recommended as a very useful tool for screening tissue sections for the presence of amyloids (Dietzel and Pfeleiderer, 1959; Puchtler and Sweat, 1965; Cooper, 1969; Romhányi, 1971; Linke, 2000). However, CRF is only specific for amyloid when controlled for green birefringence, because CRF only increases the detectability of the CR binding wherever it might occur (collagen, elastin). With this precaution, CRF can be used (1) for picking up amyloid below an immunohistochemical overlay and assuring specificity of the immunohistochemical marker (see Section 8.4), (2) for detecting amyloid in thick tissue smear that would have been missed otherwise (Figure 11.1-4), (3) for identification of amyloid deposits that are too tiny for detection by the usual CR procedure used (see Section 4.3). It is also indispensable for the exclusion of a sampling error (see Section 6.3; 6.4), and (4) for detecting low CR binding amyloids when the conventional CR procedure is evaluated as negative for various reasons. Most importantly, when serial sections are not available and the amyloid deposits are very scarce and scattered, we prestain all sections first with our quick CR staining procedure for 10–30 seconds (see Section 8.4) before we apply immunohistochemistry. By this short exposure to CR the amyloid is not stained red microscopically, and CR does not compete with the immunohistochemical chroma. Yet, the CR binding is visible when CRF is being applied. Thus, by switching the light source every immunohistochemically detectable spot can be individually judged whether or not it is congophilic. When, in addition, green birefringence can be shown, the amyloid can be verified even in very scarce and small amyloid deposits, which usually escape detection.

For these reasons, CRF adds a new dimension in sensitivity and in conjunction with the green birefringence to the precision of the diagnosis of amyloid and amyloidosis due to its high sensitivity (Puchtler and Sweat, 1965; Wolman and Bubis, 1965; Cooper, 1979; Linke, 2000). A quantitative comparison of the three methods on a large number of tissue sections in a blind fashion has revealed the following order of sensitivity by the different methods (in parentheses the number of amyloid positive sections detected among 211 small tissue sections): CR (84) < CRIC (158) < CRF (172), with CRF being the most sensitive procedure (Linke, 2000).

6.10. Concerning the Reciprocal Properties of Sensitivity and Specificity

Microscopic diagnosis of amyloid deposits requires two distinct sequential operations, as pointed out by Cooper (1969): first the identification of even the smallest amyloid deposits using a very high-sensitivity method, which could be carried out with suboptimal specificity. When, however, amyloid is suspected, let's say by CRF, it should be identified as such with a high-specificity method, that is, the search of the colored anisotropy (green birefringence in standard tissue sections). Natu-

rally, the latter method is less sensitive compared to the former. This applies even more to thioflavin S and T, because this method is reported to be unspecific for amyloid (see Section 3.7).

6.11. The Polarization Shadow

The phenomenon designed “polarization shadow” has lead to false negative results in 5 out of 211 (2.4%) tissue sections containing minute amyloid deposits examined in a blind fashion (Linke, 2000). When CR stained amyloid is evaluated in polarized light, one part of the amyloid deposit displays a bright green polarization color while the other is in the dark, showing that this portion of amyloid is, therefore, not being recognizable as such because it is invisible (**Figure 11.1-3h; see color insert**). When a small amyloid flake contains unidirectional amyloid, this could either show up by the green birefringence or be black in polarized light. To avoid a false negative diagnosis, the slide table should be turned in every section negative for amyloid, because the green illuminated amyloid between crossed Nicols moves to formerly dark amyloid areas by this procedure, and the invisible amyloid therefore becomes visible with green birefringence. Finally, the polarization shadow can easily be avoided by using CRF (see Section 6.9; Figure 11.13i; see color insert).

6.12. Inconclusiveness of a Negative Amyloid Diagnosis

The comments and results of the above sections explain why a negative bioptic amyloid diagnosis performed with a CR procedure without increased sensitivity always remains inconclusive (Michels and Linke, 1998). The comments also indicate what has to be done to improve the conventional (now obsolete, but still common) CR procedure by increasing its sensitivity (see Sections 6.8 and 6.9).

6.13. Precision of the Diagnosis and Courtesy Toward the Clinician

Reporting a negative amyloid diagnosis should contain a comment on the quality of the examined biopsy and what has been done to exclude a sampling error. The report should also contain any other technical problem. Otherwise, the clinician cannot be sure as to the validity of the reported results. This comment should include the CR procedure used, the controls, and the methods of increased sensitivity applied as well as the method that ensures the specificity before the negative diagnosis can be considered valid.

7. Chemical Identification of Amyloidosis

7.1. Before the Chemical Identification

Although this review is largely based upon the use of CR and some of its improvements, the questions concerning the chemistry of amyloid should also be summarized briefly here. Afterall, following the identification of amyloid using CR on tissues, the diagnosis of the chemical nature of amyloid is generally crucial for the precise diagnosis with respect to prognosis and finally for the treatment of the individual amyloid disease.

The first hint that amyloid is made up of chemically different amyloid “structures” came from the oxidation and digestion of amyloid in tissue sections as performed by Romhányi (1971, 1972). He demonstrated susceptible and resistant amyloid deposits, the former belonging to patients with long-standing inflammations and the latter belonging to other forms of amyloid lacking chronic

inflammations. Using a more simplified method by applying only potassium permanganate, these observations of Romhányi were confirmed and extended on a larger number of patients by Wright *et al.* (1977) and by Van Rijswijk and Van Heusden (1979), clearly confirming the distinction of the two categories identified by Romhányi. Today, this distinction is not applied anymore for clinical use because (1) it is less differential and, thus, less precise than current techniques (see below), (2) the technique is difficult to standardize (Fujihara, 1982), and (3) amyloids other than AA, such as A β_2 M, ApoAI, and ASgI, are also sensitive to oxidation (Westermarck *et al.*, 1999). This criterion, however, can prove to be ancillary when novel amyloid proteins are being characterized.

7.2. Chemical Classification

The examination of the chemical nature of amyloid proteins was pioneered by the development of techniques that were able to extract native amyloid fibrils to purity from autaptic amyloid-loaded tissues through the use of differential centrifugation and the final extraction in distilled water as described by Pras *et al.* (1968). Solubilizing and purifying the amyloid fibril proteins was achieved using high concentrations of guanidine-HCl or urea, and gel filtration, resulting in pure proteins and subsequently to the first partial amino acid sequences of two different amyloid proteins, an immunoglobulin κ -light chain by Glenner *et al.* (1971b) and the amyloid-A protein by Benditt *et al.* (1971). The letter A stands for Benditt's first amyloid protein. Later he found a non-AA type that he called amyloid-B, and subsequently proposed the first classification (Benditt *et al.*, 1972). Since then, approximately 23 different amyloid fibril proteins have been described (Westermarck *et al.*, 2002; Buxbaum, 2004), which are associated with multiple, multifarious sporadic (wild-type), and hereditary amyloid syndromes as well as a vast number of different individual amyloid diseases (Falk and Skinner, 2000; Benson, 2003; Merlini and Westermarck, 2004; Buxbaum, 2004), which, however, will not be reviewed here.

7.3. Amyloid Typing in Clinicopathologic Practice

How this accumulated knowledge has been applied for a bench-to-bedside diagnosis for the benefit of the patient will be reviewed here briefly. Most of the chemical analyses that led to the first amino acid sequences were performed using tens of grams of fresh and unfixed tissues applying autaptic tissues, making use of the macroextraction technique of Pras *et al.* (1968) or comparable methods (reviewed by Tennent, 1999). Because these methods are time consuming, expensive, and can be performed only from autopsies in some specialized laboratories, micromethods have developed starting from biopsies to distinguish the various amyloid proteins. Biopsies of various organs (see Section 6.2) were used to identify the chemical nature of the amyloid of an individual patient. Two different approaches have been developed for identifying the chemical nature of the respective amyloid in question; that is, the *immunohistochemical* and *microextraction* techniques, followed by immunochemical identification or amino acid sequencing.

7.4. Immunohistochemical Classification of Amyloids

The first immunohistochemical data directed against amyloid proteins on cryostat sections were reported by Cathcart *et al.* (1971) and Husby and Natvig (1972). Although considerable variations and various cross-reactivities have been reported, no clear distinctions of amyloid classes were noted. The first immunohistochemical analysis resulting in some distinction of different amyloid classes of 25 patients was reported by Cornwell *et al.* (1977). In addition, comparison of cryostat and fixed paraffin sections in parallel were reported to yielded similar results. Most importantly, although

anti-AA antibodies were consistent in most tissues, there was a limited reactivity noted in anti-AL antibodies with AL-amyloids (Cornwell *et al.*, 1977).

Using antibodies against isolated and well-characterized prototype amyloid proteins, satisfactory results on formalin-fixed paraffin sections were achieved by three different groups using either an anti-AA antiserum and a not further specified “anti-AL” antiserum (Levo *et al.*, 1980) or anti-AA, anti-AL λ and anti-AL κ -antibodies (Fujihara *et al.*, 1980; Linke and Nathrath, 1980; Fujihara and Glenner, 1981; Fujihara, 1982), or adding to this panel of three antibodies a fourth antibody, anti-amyloid of transthyretin origin (ATTR), after Costa *et al.* (1978) reported TTR as a new amyloid protein (Linke, 1982; Van de Kaa *et al.*, 1986; Feurle *et al.*, 1984; Dalakas *et al.*, 1984). Applying this antibody panel to tissue sections of 122 unselected patients with systemic amyloidoses, 120 (98%) could be identified and classified, demonstrating the feasibility of this approach for routine clinical amyloid typing of various amyloids (Linke *et al.*, 1984). This technique was further extended to many different amyloids using polyclonal (Chastonay and Hurlimann, 1986; Dalakas and Cunningham, 1986; Frenzel *et al.*, 1986; Kitamoto *et al.*, 1986; Allsop *et al.*, 1988) and monoclonal antibodies (Linke, 1984; Ikeda *et al.*, 1987). In addition, immunohistochemical results on the classification of amyloid were also reported on cryostat sections (Gallo *et al.*, 1986). With the discovery of more amyloid proteins this immunohistochemical option of a relatively easy and direct way of typing amyloid was extended, resulting in a panel of antibodies that could be applied to solve various questions concerning the classification of amyloidoses in patients and in retrospective lists of various tissues using several immunohistochemical variants (Donini *et al.*, 1989; Casanova *et al.*, 1992; Röcken *et al.*, 1996a, 1996b; Arbustini *et al.*, 1997; Strege *et al.*, 1998; DeCarvalho *et al.*, 2004). In particular, amyloids in biopsies of neural tissue (Feurle *et al.*, 1984; Staunton *et al.*, 1987; Li *et al.*, 1992; Jenne *et al.*, 1996), cerebral tissue (Allsop *et al.*, 1988; Kitamoto *et al.*, 1987; Baron *et al.*, 1988; Schröder *et al.*, 1995; Schröder and Linke, 1999), carpal tunnel tissue (Stein *et al.*, 1987; Kyle *et al.*, 1992), endomyocardial bioptic tissue (Frenzel *et al.*, 1986), lymph node tissue (Newland *et al.*, 1986), laryngeal tissue (Godbersen *et al.*, 1992), skin tissue (Bieber *et al.*, 1988; Dithmar *et al.*, 2004), and subcutaneous tissue (Orfila *et al.*, 1986) have been classified on formalin-fixed paraffin sections. The identification of amyloids can also be performed using peptide antibodies (Westermarck *et al.*, 1987, 1999; Solomon *et al.*, 2003a).

Care should be taken not to overlook a combination of two or more different amyloid diseases. Using immunohistochemistry, a combination of two or more different amyloid diseases can be identified easily because every amyloid reacts only with one of the different antibodies applied. As can be seen in **Figure 11.1-6; see color insert**, the two amyloids are located at different anatomical sites, indicating that the different amyloid deposits are usually not mixed even when the same anatomical structures are affected. They originate from two different diseases, that is in this case A β_2 M, as a sequel of chronic hemodialysis, which appeared first, and subsequently AA, which followed a suppurative chronic inflammation that occurred during the treatment by hemodialysis. The order of appearance is morphologically reflected in the A β_2 M globes, which seem to have grown undisturbed first followed by the AA deposits filling out the gaps between the A β_2 M deposits in line with the clinical course of the two diseases (see Section 8.6). Several combinations of different amyloids can appear in the same patient as reported (Newland *et al.*, 1986; Linke *et al.*, 1988; Störkel and Sturer, 1989; Isobe *et al.*, 1996; Bergström *et al.*, 2004).

Moreover, tissue embedding in hydroxyethylmethacrylate for light microscopy achieved excellent results (Donini *et al.*, 1984). Finally, immunoelectron microscopic classification of different amyloids using, in part, various embedding media such as hydroxyethylmethacrylate, maraglass, lowicryl, and epon using monoclonal and polyclonal antibodies, was achieved with clear results (Linke *et al.*, 1983b; Donini *et al.*, 1984; Orfila *et al.*, 1986; Ikeda *et al.*, 1987; Linke *et al.*, 1989; Arbustini *et al.*, 1997, 2002), demonstrating an increased signal-to-background staining due to the

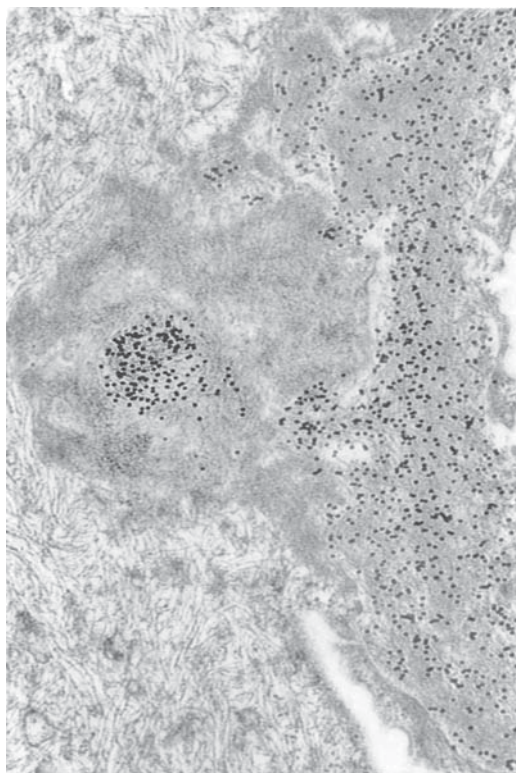


Figure 11.1-5. Electronmicroscopic typing of amyloid. Renal basement membrane amyloid is labeled with our murine anti-AA(mcl) monoclonal antibody (Linke, 1984) and gold colloid particles of 17-nm diameter. Only the amyloid is clearly and specifically stained. There is virtually no background staining, and the intermediate filaments in podocytes (left-hand side) and endothelial cells (right margin) are not labeled, thus illustrating the high sensitivity and specificity of immunoelectron microscopy (Linke *et al.*, 1989)

resistance of the amyloid fibril towards processing-induced denaturation while the unspecific binding is destroyed. Figure 11.1-5 demonstrates the clear and specific immunoelectron microscopic staining pattern of amyloid fibrils, while unspecificity is lacking.

Immunohistochemistry has also been extended to animal amyloids by applying antibodies against human amyloids with crossreactivity toward animal amyloids or the reverse, suggesting, in part, some common amyloid conformations in different proteins (Kitamoto *et al.*, 1986; Van de Kaa *et al.*, 1986; Allsop *et al.*, 1988; Zschiesche and Linke, 1989; Colbatzki *et al.*, 1991; Platz *et al.*, 1997; Ofri *et al.*, 1997; Schulz *et al.*, 1998; Majzoub *et al.*, 2003).

There are, however, some problems that need to be addressed, that is: (1) with the immunohistochemical identification of the different amyloids when antibodies are being used that are directed against native proteins. So, Chastanay and Hurrelimann (1986) reported that only half of the AL-amyloidoses can be identified with antibodies directed against native light chains, which is the experience of many groups including our own. Based on these findings we only used our own antibodies

raised against either amyloid proteins or peptides (Linke, 1982, 1987, 2000; Linke *et al.*, 1984; DeCarvalho *et al.*, 2004) with consistent results. The reason for the described properties of anti-amyloid antibodies is discussed in Section 8.2. (2) Another point refers to cryostat versus paraffin sections. As shown by Cornwell *et al.* (1977), both kinds of biopsies can be successfully used. Comparison of the two resulted in a higher specificity in paraffin sections (Linke and Nathrath, 1980; see Table 11.1-1). In addition, our experience with cryostat sections was that they are difficult and more expensive to get, more expensive to store, and that retrospective studies are problematic because most tissues are preserved in the form of fixed tissues in paraffin blocks. We, therefore, use only paraffin sections with excellent results since 1979. (3) Amyloids can only be detected when an appropriate antibody is available (see Section 8.3). Otherwise, misdiagnosing amyloids could be the result (Lachmann *et al.*, 2002). Therefore, only a complete set of antibodies can exclude the other possible candidates (Linke, 2000; DeCarvalho *et al.*, 2004). (4) In addition, although pretreatment of the tissue sections before immunohistochemistry has been shown to be useful in cerebral amyloids using particular antibodies (Kitamoto *et al.*, 1987), visceral amyloid in our system does not need pretreatment in most cases, and may even harm the results. (5) When amyloids cannot be classified with a panel of proven antibodies using immunohistochemistry novel amyloids classes need to be considered. All these unknown cases need first biochemical analysis followed by the preparation of new antibodies, which then have to be tested on a larger series of positive and negative controls to ensure their specificity and correct performance (see Section 7.5).

7.5. Microextraction Followed by an Amino Acid Sequence

Another approach to identify the amyloid proteins for clinical use in patients is miniaturizing the extraction method of unfixed fresh tissues. Microextraction modifying the Pras' method on native biopsies with the aid of 10–30 µg of tissue biopsies followed by immunochemical identification of the purified amyloid fibril proteins resulted in reliable typing of various amyloids (Linke and Nathrath, 1980; Turner *et al.*, 1983) or amino acid sequencing of the purified amyloid proteins (Westermarck *et al.*, 1989; Custano *et al.*, 1997; Kaplan *et al.*, 1997). Selective extraction of AA-protein from formalin-fixed tissue was pioneered by Shtrasburg *et al.* (1982). This unusual property of amyloid proteins seems to be due to the fact that they are not being crosslinked by formaldehyde while most other proteins are. This behavior of amyloids is in line with their resistance toward enzymatic degradation, their resistance towards solubilization (Glenner *et al.*, 1974), and their resistance towards fixation-induced denaturation of antigenic determinants (Linke *et al.*, 1989, see Table 11.1-1). Amyloid proteins extracted from formalin-fixed tissues can also be amino acid sequenced (Linke *et al.*, 1983a; Layfield *et al.*, 1996). Layfield *et al.* (1997) proposed fixation as a means even to purify the amyloid proteins. They also proposed a special tight packing of the polypeptide chains within the amyloid fibrils that protects the polypeptides from chemical attack. Interestingly, Balbirnie *et al.* (2001) demonstrated “a dehydrated β -sheet structure” in amyloid-like fibrils as derived from the yeast protein Sup35. This is in line with a reduced D₂-exchange of the core of the amyloid-like fibrils and even the protofibrils as reported by Kheterpal *et al.* (2000, 2003). As it seems, the amyloid may be so tightly packed that the core cannot react because it is “dry,” bringing to mind the proverb of Paracelsus “*corpora non agunt nisi soluta*” (only dissolved substances can react).

Today, amyloid proteins can even be microextracted and microsequenced from formalin-fixed paraffin sections, as reviewed in detail by Kaplan *et al.* (1999), which was even further examined and applied by Kaplan *et al.* (2001), Murphy *et al.* (2001), Solomon *et al.* (2003a), and Yazaki *et al.* (2004). Moreover, the results of the microsequencing technique have been shown to be in accordance with immunohistochemical data on a limited number of patients (Kaplan *et al.*, 2004). In addition,

Kaplan *et al.* (1999) pointed out that the microsequencing technique is not trivial because other proteins are extracted together with the amyloid proteins. Therefore, it should be proven in every case which of the extracted proteins is indeed the amyloid fibril protein in question. This is particularly important when novel amyloid proteins are being identified (Solomon *et al.*, 2003a; Linke *et al.*, 2004). Finally, when concomitant proteins can be well discerned, clear diagnoses can be achieved and amyloid classification has then gained a new degree of precision. This should especially be the case when larger amounts of amyloid are present in tissue sections. Whether the same classification can also be performed when very small amyloid deposits are present, that is early in the course of the disease, needs still to be explored.

8. Advice for the Immunohistochemical Classification of Amyloids

8.1. Introduction

The immunohistochemical classification of amyloids is not trivial, because it implies the knowledge of the technique, how to overcome the pitfalls by quality checks, and the correct evaluation based on experience before it can be applied with reliability. One should also consider some of the background work that made the consistent and specific reactivity of anti-amyloid antibodies possible, because in many institutes immunohistochemistry has not been developed to the state of reliability on a large panel of different amyloids. The reason why antibodies against native light chains do not in all cases react with light-chain amyloids has been explored using an *in vitro* model. The formation of amyloid-like fibrils from BJP *in vitro* was pioneered by Glenner *et al.* (1971) using pepsin in acid. We have changed the digestion protocol to trypsin because this method yielded a stable preamyloidotic BJP fragment that could be column purified and transformed to amyloid-like fibrils in seconds upon acidification. Comparing antibodies directed against the native BJP versus a soluble preamyloidotic tryptic fragment of a λ -BJP has suggested the answer. The finding that amyloidogenesis is a two-step procedure (Linke *et al.*, 1973) has pathogenetic significance. The reactivity of the two antibodies with the three different antigens shows that antibodies against the native BJP having specificity against the V- and the C-region reacted neither with the soluble preamyloidotic fragment (although it was derived from the V-region) nor with its acidic product, the amyloid-like fibrils, and reversely, the antibody against the preamyloidotic precursor reacted only against the homologous immunogene and its acidic product, the amyloid-like fibrils, but not with the native protein of origin. The antibody against the preamyloidotic fragment can, therefore, be considered as amyloid-specific based on a defined and stable conformation of the soluble monomeric preamyloidotic precursor. It did, however, react only with the BJP after reduction and alkylation, indicating a profound conformational change of the native BJP with the exposure of hidden antigenic determinants during the amyloidogenic transformation including the loss of the native idiootype from the V-fragment (Linke *et al.*, 1973). Conformational change during the amyloidogenic transformation has been described in numerous systems using modern techniques (Sunde and Blake, 1998; Dobson, 2003), and amyloid-specific antibodies that are central to amyloid therapy today have been reviewed by Glabe (2004).

8.2. Approved Antibodies and Controls

Our antibodies are prepared according to the principles discussed above (Section 8.1). Only those antibodies that have been shown to detect all members of the respective amyloid class on a

panel of many tissues should be used. In addition, these antibodies should not interfere with other classes. We use only our own antibodies because the immunogenes are known, and their performance has been assured by tests on hundreds of different tissue sections. Because not all immunohistochemical tests result in the same staining intensity of the amyloid deposits, every antibody must be tested in the same assay on a tissue section of the same amyloid class to ensure the reliable performance of a given antibody in a particular test as a positive control.

8.3. Sets of Antibodies

We use a set of a larger panel of different antibodies in the first run, which identifies at least 95–98% of all amyloids submitted from physicians for routine classification (Linke, 1987; DeCarvalho *et al.*, 2004). These antibodies are directed against AA (multiple), also multiple AL λ -, AL κ -, and AH γ -antibodies, ATTR, A β ₂M, AFib, AApoAI, AApoAII, and ALys. The latter are being used in particular in the hereditary varieties of amyloidosis. In cerebral amyloids we add antibodies recognizing A β (several), ACys, and APrion antibodies (Schröder *et al.*, 2000). Others are in preparation. When local and organ-limited amyloids are suspected we add reagents against a whole list of local or organ-limited amyloids to make sure that these amyloids are of limited distribution in a given patient to provide solid information for the patient as to the prognosis of a given disease.

In cases in which an animal amyloid needs to be classified, we use mostly antibodies directed against human amyloids that crossreact with the respective animal amyloids in tissue sections (some AL λ and some AA antibodies seem to be generic for most or all AL λ or AA amyloids), that include for AL λ such species as the horse, dog, cat, and humans, and for AA, all tested mammalian and avian species tested so far (Geisel *et al.*, 1990; Colbatzky *et al.*, 1991; Ofri *et al.*, 1997; Platz *et al.*, 1997; Schulz *et al.*, 1998; Zschesche and Linke, 1989).

8.4. Prestaining with CR

When amyloid deposits show the sampling error (see Section 6.3), all available 15–20 paraffin tissue sections will be stained with CR first and the sections containing amyloid selected for immunohistochemistry. The congruence of the immunohistochemical marker can then be compared with the amyloid as identified using CRF (see Section 6.9). With this double staining and the increased sensitivity using CRF the assignment of the immunohistochemically stained spots to the amyloid is easily achieved because the immunohistochemical chromogene does not interfere with the CRF (see Section 6.9).

8.5. Serial Sections

When amyloid deposits are small, scarce, and/or situated at irregular sites, so that they may escape recognition on nonserial sections, serial sections are very useful to recognize these spots immediately from section to section and find out the minute spots using knowledge gained from the section above or below with a larger amyloid spot. The gain in size and the fading of an amyloid spot from cut to cut tremendously improves the recognition of very small amyloid deposits because one can concentrate the inspection and evaluation onto a single spot using maximal magnification. When, however, serial sections are not available, all tissue sections need to be prestained with CR and processed as discussed in Section 8.4. In addition, we also prefer using serial sections for photographic documentation of the different immunohistochemical results for improved morphologic comparison of the results (**Figure 11.1-3g–m; see color insert; see Section 7.4**).

8.6. Other Amyloid Components

The evaluation also needs a comment on the expected and finally obtained immunohistochemical reactions, which needs to take into account the fact that the amyloid deposit in tissue is not a pure substance (Linke, 1987), but a deposit associated with a host of different constituents (Snow *et al.*, 1987; Strittmatter and Roses, 1996; Kisilevsky and Fraser, 1997; Kisilevsky, 2000); some are part of the fibril and others are not. In addition, the amyloid is being perfused by many soluble proteins that can be adsorbed to the amyloid fibril, the most common being the amyloid P component, which has been detected virtually in all amyloids examined to date, and which may have a pathogenetic significance (Pepys *et al.*, 1997; Tennent, 1999; Pepys, 2001). The immunoglobulin λ -light chain, which is detectable in varying amounts in many amyloids for unknown reasons so far (Fujihara and Glenner, 1981; Linke, 1982, 1987; Linke *et al.*, 1984), seems to be adsorbed to the fibril and may confuse the diagnosis when the respective antibody that is needed for the identification of the nature of the amyloid protein in question is not available (Lachman *et al.*, 2002). It is, therefore, important to exclude all possible amyloids when an anti-AIg reactivity is present. This is only possible when a whole set of proven anti-amyloid antibodies is available.

It is also most important to recognize when more than one amyloid is present in a patient because the prognosis and therapy may be misleading when only one of the two (or more) amyloids is considered for therapy and the other possibly more dangerous amyloidosis may not even be recognized (see Section 7.4).

9. From Bench to Bedside: An Algorithm for a Reliable Diagnosis

9.1. Classifications Before the Chemical Nature of Amyloid Was Known

Different strategies have been applied to categorize the various amyloid diseases. Before the chemical nature of amyloid was known, these categories were based on clinical associations, organ distribution, and hereditary characteristics (Lubarsch, 1929; Reiman *et al.*, 1935; Isobe and Osserman, 1974). These categories were inconsistent because they showed many overlaps and no clear distinction could be made for therapeutical considerations, except for the category “secondary” or inflammation-induced amyloidosis with the various reactive and hereditary forms as FMF and MWS (Zemer *et al.*, 1986). The rest was referred to as “primary” amyloidosis meaning amyloid “without inflammation.” We know that the latter category can comprise almost every amyloid class known today. Therefore, the usage of the ambiguous terms “primary” or “secondary” in designating amyloid syndromes was discouraged with the advent of chemical terms at the Third International Symposium on Amyloid an Amyloidosis in Póvoa de Varzim, Portugal, 1979.

9.2. Classification According to the Chemical Nature of Amyloid Proteins

With the advent of the chemical identification of *ex vivo* amyloid proteins, a new nosology has emerged as designed by Benditt and Eriksen (1972) and by Glenner (1980). This amyloid classification based on the chemistry of the amyloid deposits in patients led to consistent amyloid diseases and amyloid syndromes with a rational nomenclature (Westermarck *et al.*, 2002; see also Abbreviations). Based on these amyloid categories therapies have emerged that are already successful in some of the amyloid syndromes (see Section 2).

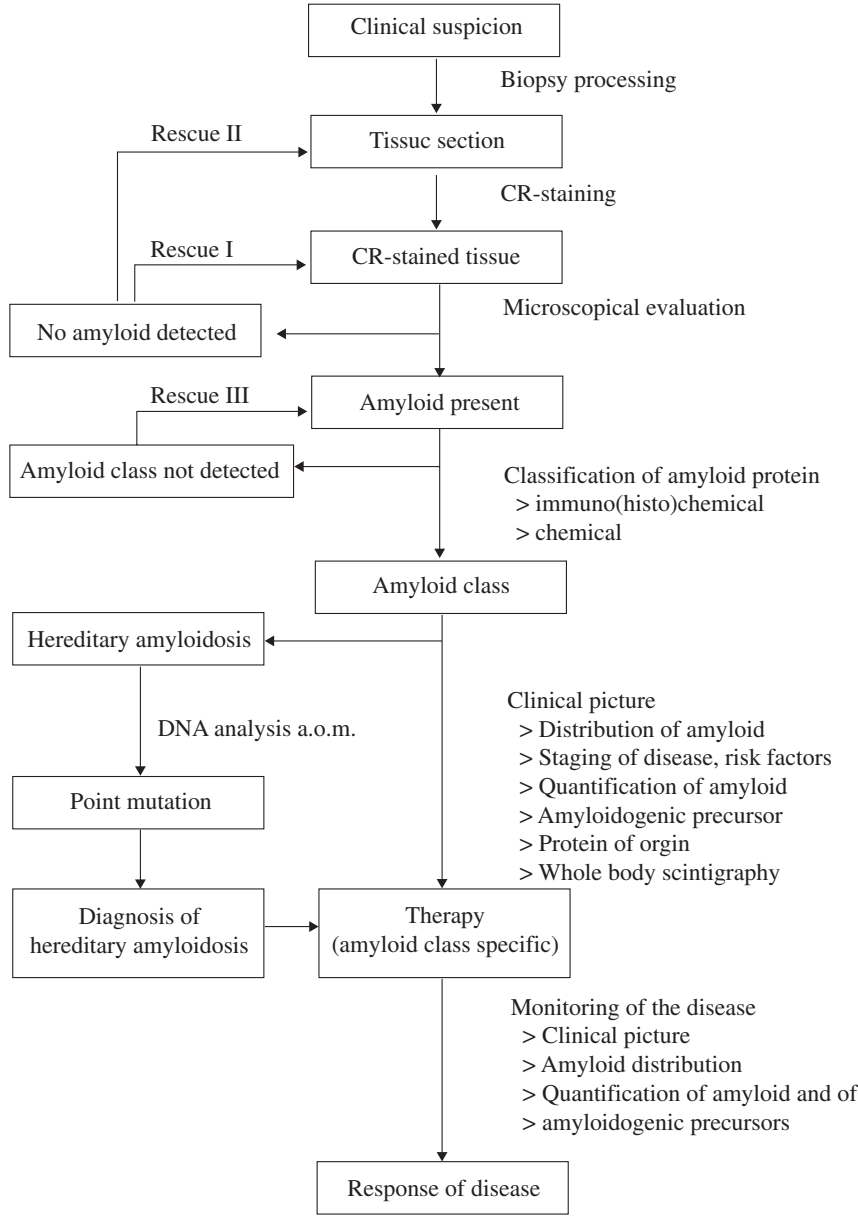


Figure 11.1-7. Bench-to-bedside diagnosis: An algorithm for diagnosing and treating amyloidoses (see Sections 9.3 and 9.4)

9.3. An Important Remark Pointing to the Correct Hierarchy of Diagnosing Amyloidosis

To arrive at a precise diagnosis today does *not primarily* include the identification of one of the soluble precursors, because only the amyloid can tell which of the many different precursors has finally produced the amyloid in question (Glenner, 1980; Linke *et al.*, 1998; Murphy *et al.*, 2001; Merlini and Westermark, 2004). The soluble precursors are only the risk factors, as exemplified in an elevated SAA, in the presence of a monoclonal protein in serum and/or in urine, or a mutated serum protein in hereditary amyloidoses such as TTR. Although it is likely that these precursors are the origin of the amyloid in each of the amyloidoses, the patient's amyloid has to be identified in each case. Only this information will tell which of the many possible proteins in plasma and tissues is indeed the protein of origin of this particular amyloid in a given patient (Glenner, 1980). How to diagnose the various amyloidoses on the basis of the chemistry of the deposited protein is illustrated in Figure 11.1-7, demonstrating a step-by-step approach as in the form of a flow sheet, which is an extended version of the recently published flow sheet (Linke *et al.*, 1998).

9.4. Diagnosing Amyloidosis Correctly with Respect to Therapy

To diagnose the class of amyloidosis, one needs to analyze *the deposited amyloid* and not primarily one of the many soluble precursors, because only the amyloid in tissue defines which of the many precursor has induced the amyloid and consequently causes the disease in question (Benditt *et al.*, 1972; Glenner, 1980; Linke *et al.*, 1998; Murphy, 2001; Merlini and Westermark, 2004; Buxbaum, 2004). The following overall flow sheet in Figure 11.1-7 illustrates the different steps that can be followed to arrive at a precise diagnosis.

The *suspicion of the clinician* is the most decisive first step and starting point, because the earlier the diagnosis of amyloidosis is made, the more favorable will be the prognosis for the patient. The diagnosis of amyloidosis has to be considered by such symptoms as unexplained sudden weight loss, fatigue, malabsorption, polyneuropathy, unexplained renal disease, hepatosplenomegaly, macroglossia, carpal tunnel syndrome, monoclonal gammopathy, chronic inflammation, intestinal problems, hemorrhagic and other related diseases, hereditary syndromes, dementia, congestive heart failure, joint problems, and many other symptoms. The safest diagnosis of amyloidosis is brought about though a *biospy* (see Section 7.4), which is taken either from subcutaneous fatty tissue or from such organs as the intestine, including the rectum, kidney, liver, or heart, and many other sites (Cohen and Calkins, 1959; Gafni and Sohar, 1960; Glenner, 1980; Westermark *et al.*, 1989; Falk and Skinner, 2000; Merlini and Westermark, 2004). However, when amyloid could not be detected, the Rescue I program (see Figure 11.1-7) is put into force. This program will exclude the sampling error by examination of additional sections (see Section 6.3) and increase the sensitivity of the CR procedure by CRF (see Section 6.9) and, when needed, by immunohistochemistry (see Sections 6.8 and 8). In case no amyloid can also be detected using the Rescue I program, Rescue II will be enacted, which includes the taking of additional biopsies that are examined likewise. In case no amyloid can be detected, one should be aware of nonamyloidotic *protein deposit diseases* (Gallo *et al.*, 1980; Picken *et al.*, 1989; Buxbaum, 1992; Walker and LeVine, 2000), which are immunohistochemically examined the same way as amyloid. A negative diagnosis of amyloid should not be made without having followed the program of Rescue I and II (see Section 6.12).

When *amyloid is detected* via the biopsy, the next step will be to find out its chemical nature. Because the detection of soluble amyloid precursors is not safe, the amyloid itself needs to be examined (Glenner, 1980; Kaplan *et al.*, 1999; Murphy *et al.*, 2001). The easiest method for this aim involves *immunohistochemistry*, which is assumed to be available in most institutes due to its easy performance, and which has been performed on both fixed paraffin and plastic sections for light and

electron microscopy (see Section 7.4). In addition, chemical *microextraction methods* can be applied to native or fixed tissue and to fixed tissue from paraffin sections (see Section 7.5). In case the amyloid class cannot be determined with certainty, Rescue III is put into operation. When autolysis and/or imbibition is the main obstacle, a freshly taken biopsy should be handled with care (see Section 6.6) and examined likewise. Another possibility is to buy, prepare, and try additional antibodies. In this case, microextraction techniques (Section 7.5) could also be successful when available. On the other hand, when amyloid is too scarce for microextraction procedures, immunohistochemistry may lead to the desired result. When amyloid is very scarce, the sections should be prestained with CR before being immunohistochemically examined (see Sections 6.8, 6.9, and 8.4). Finally, nonamyloidotic protein deposit diseases can also be classified with anti-amyloid antibodies (Casanova *et al.*, 1992).

When the *amyloid class is known*, the amyloid syndrome is identified and the possible prognosis and the possible therapeutic options can be considered from the biology of the precursor if known. When a syndrome is hereditary, the respective genes are to be sequenced, and the point mutation (or the point mutations) are to be identified (Benson, 1995, 2003; Falk and Skinner, 2000; Pepys, 2001; Buxbaum, 2004). The different therapies will be monitored by various measures (see Figure 11.1-7) to document the response, the partial response, or the lack of it. Rescue IV (not in Figure 11.1-7) would be valid when an unknown amyloid protein is present. This situation needs chemical analysis (see Sections 7.2-7.5 and 8), the most elegant method being microextraction (Kaplan *et al.*, 1999) and proof as to which of the proteins extracted from formalin tissue is indeed the amyloid protein (Solomon *et al.*, 2003a; Linke *et al.*, 2004).

10. Quantification of Amyloid

This is only a short remark. There is currently a debate concerning whether or not the amount of amyloid is indeed the most important factor causing the clinical symptoms, because the most toxic molecular species could be represented *in vitro* by the preamyloidotic oligomers (reviewed by Buxbaum, 2004). Three methods are in use today for the quantification of amyloid in patients: (1) the morphometric measurement of amyloid in tissue sections with a numerical readout, (2) the whole-body scintigraphy with radioactive tracers without a numerical readout, and (3) the exact quantification of amyloid proteins in solubilized form from biopsies that shows a good correlation with the clinical situation (Hazenbergh *et al.*, 1999), which would still require a bioptic controls.

Morphometric quantification of amyloid in tissue sections was reported on the light microscopic level (Donini *et al.*, 1991; Royston *et al.*, 1994) and on the electron microscopic level (Takei *et al.*, 2001). There are, however, some problems that should be addressed, one being the selection of the organ to be analyzed. Large sections of homogeneous organs as liver (Michels *et al.*, 1994) or brain tissue (Royston *et al.*, 1994) do not pose a problem because representative areas can easily be selected or the data averaged by sampling of many different areas. The situation, however, changes when only small biopsies are available because amyloid deposits are very variable in several organs and tissues, and the amyloid in the biopsy may not be representative. Because the amyloid might be deposited in irregular fashion, one should prefer organ structures that are relevant for the prognosis of the patient to correlate the amount of amyloid with the course of the disease. We have chosen the kidney and the liver for morphometric quantification. The renal glomerulus was shown to represent an ideal organ structure for this quantitation, because it is encircled with a capsule and the amyloid in the glomerular tufts is relevant for the prognosis (Donini *et al.*, 1991). As an example, the quantity of amyloid was morphometrically measured in the glomeruli of two patients with AL λ amyloidosis, both before and 3 years after autologous stem cell transplantation. Both patients had a complete remission and the clinical situation improved significantly, but the amount of amyloid was seen to remain the same after 3 years, because the small increase in one patient was statistically not relevant

(Zeier *et al.*, 2003). These data demonstrate that the mature glomerular amyloid is not the only decisive factor leading to amyloid nephrosis and finally organ failure in the patient. Because the proteinuria improved significantly and the amyloid remained the same, this amyloid did not cause the proteinuria. Moreover, the liver is also an ideal organ for quantification when amyloid is deposited as capillary amyloid. As an example, liver amyloid of the AA-type was quantitated morphometrically in a patient with juvenile rheumatoid arthritis before therapy and during a consequent anti-inflammatory therapy. Examining three consecutive liver biopsies, it could be documented that the area of amyloid of 17% before therapy was reduced to below 2% within a period of approximately 6 years during therapy (Michels *et al.*, 1994). Moreover, Takei *et al.* (2001) reported the amount of amyloid of sural nerve biopsies in six FAP patients before and 3 years after liver transplantation. They showed neither regression nor significant progression, stating that this result is an objective measure showing that the accumulation of amyloid had stopped after liver transplantation. Finally, the precision of four different makers of amyloid for a quantitative readout have been compared, that is CR, green birefringence, CRIC, and CRF. The latter yielded the most precise and most consistent values due to the high sensitivity of the bright fluorescence. CRF can therefore be recommended as an ideal marker for morphometric measurements of amyloids (Donini and Linke, 2004). However, it should be mentioned again that CRF is only specific when controlled microscopically through the green anisotropy, as seen in polarized light (see Section 6.9).

After the amyloid has been detected and classified, one would like to know the extent of the organ involvement for therapeutic considerations and the subsequent monitoring of the disease activity in some patients. To this end, an inexpensive and reliable method for whole-body imaging of the amyloid would be desirable. However, all tracers so far available are still a matter of debate for various reasons. The most prominent tracer today is the amyloid-P-component (AP), a normal serum protein (SAP), which is found to be present in all amyloid deposits so far analyzed. The use of radioactive SAP for all amyloids in animals and patients has been summarized (Hawkins, 1994). Another tracer is the radioactive diphosphonate DPD for imaging only ATTR in patients suffering from FAP and SSA. Differently from the pattern received by SAP, which almost exclusively traced the liver, the DPD images ATTR amyloid of heart, nerves, bowel, and skin, but not of the liver, in accordance with the quantity of amyloid in these organs (Puille *et al.*, 2002). In addition, the CR analogue chrysamine G has been used, in comparison with SAP (Dezutter *et al.*, 2001), for radioactive imaging of the articular and systemic AA-amyloid in infected chickens (see Section 3.8). Finally, there exists an intensive effort to diagnose the cerebral A β amyloid in Alzheimer's disease and congophilic amyloid angiopathy before the "point of no return" of the dementia has been reached, to try to arrest or even prevent this inexorably progressive disease. This effort is still in its early stage as exemplified by some citations including the application of CR and thioflavin analogues, and other substances (Klunk *et al.*, 2001, 2002, 2003a,b; Bacskai *et al.*, 2002, 2003; Wiesehan *et al.*, 2003; Wang *et al.*, 2004).

11. Novel Techniques in Amyloid Research

Although this review describes the use of CR in diagnosing amyloid based on its high specificity, some other applications using the same property will be mentioned here briefly. It has been shown that CR cannot only bind to amyloid and amyloid-like fibrils, but can also influence the formation of amyloid *in vitro* and alter its toxicity in living systems. Thus it has been shown that CR can inhibit neurotoxicity (Lovenzo and Yanker 1994) and can protect hippocampal neurons against A β cytotoxicity (Burgevin *et al.*, 1994). Similarly, Chrysamine G can inhibit A β -induced cytotoxicity in tissue culture (Klunk *et al.*, 1998) and neurotoxicity in mice (Ishii *et al.*, 2002). In addition, the formation

of prion peptide-derived amyloid-like fibrils can be inhibited *in vitro* using CR analogues (Rudyk *et al.*, 2003; Sellarajah *et al.*, 2004) and by trypan blue, sirius red, and other CR analogues (Demaimay *et al.*, 2000). Moreover, the cytotoxic drug deoxydoxorubicin was reported to bind to amyloid *in vivo* and *in vitro*, and can alter the course of AL-amyloidosis in some patients (Merlini *et al.*, 1999; Cardoso *et al.*, 2003). These properties of agents that bind and reduce the toxicity of amyloid proteins and their precursors may represent a basis for the development of novel drugs against various amyloid diseases. Finally, an enormous effort has been made, and success has appeared in some cases by using antibodies in experimental *in vitro* conditions, and antibodies and immunizations in animals and transgenic animals and humans (reviewed by Solomon *et al.*, 2003b; Buxbaum, 2004), using antibodies in general, amyloid-specific antibodies (reviewed by Glabe, 2004), and generic antibodies, which recognize all amyloids alike irrespective of their chemical composition (O'Nuallain and Wetzel, 2002; Solomon *et al.*, 2003b).

12. Abbreviations

AA	amyloid-A protein
AL λ /AL κ	amyloid of the immunoglobulin λ / κ light chains origin
AIg/AH γ	amyloid of immunoglobulin origin/amyloid of immunoglobulin γ heavy chain origin
A β_2 M	amyloid of beta $_2$ -microglobulin origin
AFib	amyloid of fibrinogen origin
ApoAI/II	amyloid of apolipoprotein I/II origin
ALys	amyloid of lysozyme origin
ASgI	amyloid of semenogelin I origin
A β	amyloid beta protein (in the Alzheimer's disease complex)
APrion	amyloid proteins in prionoses
ATTR	amyloid of transthyretin origin
CR	Congo red
CRF	Congo red fluorescence
CRIC	Congo red and immunocytochemistry
FAD	Familial amyloid polyneuropath
SSA	Senile systemic amyloidosis
SAP	Serum amyloid P-component

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References

- Abraham, R.S., Katzmann, J.A., Clark, R.J., Kyle, R.A., and Certz, M.A. (2003). Quantitative analysis of serum free light chains. A new marker for the diagnostic evaluation of primary systemic amyloidosis *Am. J. Clin. Pathol.* 119:274–278.

- Allsop, D., Ikeda, S., Bruce, M., and Glenner G.G. (1988). Cerebrovascular amyloid in scrapie-affected sheep reacts with antibodies to prion protein. *Neurosci. Lett.* 92:234–239.
- Arbustini, E., Morbini, P., Verga, L., Condardi, M., Porcu, E., Pilotto, A., Zorzoli, I., Garini, P., Anesi, E., and Merlini, G. (1997). Light and electron microscopy immunohistochemical characterization of amyloid deposits. *Amyloid J. Protein Fold. Disord.* 4:157–170.
- Arbustini, E., Verga, L., Condardi, M., Palladini, G., Obici, L., and Merlini, G. (2002). Electron and immunoelectron microscopy of abdominal fat identifies and characterizes amyloid fibrils in suspected cardiac amyloidosis. *Amyloid J. Protein Fold. Disord.* 9:108.
- Bacskaj, B.J., Klunk, W.E., Mathis, C.A., and Hyman, B.T. (2002). Imaging amyloid-beta deposits in vivo. *J. Cereb. Blood Flow Metab.* 22:1035–1041.
- Bacskai, B.J., Hickey, G.A., Skoch, J., Kajdasz, S.Z., Wang, Y., Huang, G.F., Mathis, C.A., Klunk, W.E., and Hyman, B.T. (2003). Four-dimensional multiphoton imaging of brain entry, amyloid binding, and clearance of an amyloid-beta ligand in transgenic mice. *Proc. Natl. Acad. Sci. USA* 100:12462–12467.
- Balbirnie, M., Grothe, R., and Eisenberg, D.S. (2001). An amyloid-forming peptide from the yeast prion Sup35 reveals a dehydrated beta-sheet structure of amyloid. *Proc. Natl. Acad. Sci. USA* 98:2375–2380.
- Baron, H.A., Evercooren, B.V., and Brucher, J.M. (1988). Antiserum to scrapie associated fibril protein reacts with amyloid plaques in familial transmissible dementia. *J. Neuropathol. Exp. Neurol.* 47:158–165.
- Bellotti, V., and Merlini, G. (1996). Current concepts on the pathogenesis of systemic amyloidosis. *Nephrol. Dial. Transplant.* 11(Suppl. 9):53–63.
- Ben-Chetrit, E., and Levy, M. (1991). Colchicine prophylaxis in familial mediterranean fever. Reappraisal after 15 years. *Semin. Arthritis Rheum.* 20:241–246.
- Benditt, E.P., and Eriksen, N. (1972). Chemical classes of amyloid substance. *Am. J. Pathol.* 65:231–249.
- Benditt, E.P., Eriksen, N., Hermosden, M.A., and Ericsson, L.H. (1971). The major proteins of human and monkey amyloid substance: common properties including unusual N-terminal amino acid sequences. *FEBS Lett.* 19:169–173.
- Bennhold, H. (1922a). Über die Ausscheidung intravenös einverleibter Farbstoffe bei Amyloidkranken. *Verh. Deutsch. Ges. Inn. Med.* 34:313–315.
- Bennhold, H. (1922b). Eine spezifische Amyloidfärbung mit Kongorot. *Münchn. Med. Wochenschr.* 69:1537–1538.
- Bennhold, H. (1923). Über die Ausscheidung intravenös einverleibten Kongorots bei den verschiedenen Erkrankungen, insbesondere bei Amyloidose. *Deutsch. Arch. Klin. Med.* 142:32–46.
- Benson, M.D. (1995). Amyloidosis. In: Sciver, C.R., Beaudet, A.L., Sly, W.S., and Valle, D. (eds.), *The metabolic basis of inherited diseases*, vol. 3. New York: McGraw-Hill, pp. 4139–4191.
- Benson, M.D. (2003). The hereditary amyloidoses. *Best Pract. Res. Clin. Rheumat.* 17:909–927.
- Bergström, J., Murphy, C.L., Weiss, D.T., Solomon, A., Sletten, K., Hellman, U., Westermark, P. 2004. Two different types of amyloid deposits apolipoprotein A-IV and transthyretin – in a patient with systemic amyloidosis. *Lab Invest.* 84:981–988.
- Bieber, T., Ruzicka, T., Linke, R.L., van Kies, R., Goez, G., and Braun-Falco, O. (1988). Hemorrhagic bullous amyloidosis. A histologic, immunohistochemical and ultrastructural study of two patients. *Arch. Dermatol.* 124:1683–1686.
- Bonar, L., Cohen, A.S., and Skinner, M.M. (1969). Characterization of the amyloid fibril as a cross- β protein. *Proc. Soc. Exp. Biol. Med.* 131:1373–1375.
- Burgevin, M.C., Passat, M., Daniel, N., Capet, M., and Doble, A. (1994). Congo red protects against toxicity of beta-amyloid peptides on rat hippocampal neurons. *Neuroreport* 5:2429–2432.
- Buxbaum, J.N. (1992). Mechanism of disease: monoclonal immunoglobulin deposition. Amyloidosis: light chain deposit disease, and light and heavy chain deposition disease. *Hematol. Oncol. Clin. North Am.* 6:323–346.
- Buxbaum, J.N. (2004). The systemic amyloidoses. *Curr. Opin. Rheumatol.* 16:67–75.
- Buxbaum, J.N., and Tagoe, C.E. (2000a). The genetics of the amyloidoses. *Annu. Rev. Med.* 51:543–569.
- Buxbaum, J.N., Genega, E.M., Lazowski, P., Kumar, A., Tunick, P.A., Kronzon, I., and Gallo, G.R. (2000b). Infiltrative nonamyloidotic monoclonal immunoglobulin light chain cardiomyopathy: an underappreciated manifestation of plasma cell dyscrasias. *Cardiology* 93:2290–2298.
- Caesar, R. (1960). Die Feinstruktur von Milz und Leber bei experimenteller Amyloidose. *Z. Zellforsch. Mikrosk. Anat.* 52:653–673.
- Calkins, E., and Cohen, A.S. (1960). Diagnosis of amyloidosis. *Bull. Rheum. Dis.* 10:215–218.
- Carnes, W.H., and Focker, B.R. (1956). The metachromasy of amyloid. *Lab. Invest.* 5:21–43.
- Cardoso, I., Merlini, G., and Saraiva, M.J. (2003). 4'-iodo-4'-deoxydoxorubicin and tetracyclines disrupt transthyretin amyloid fibrils in vitro producing noncytotoxic species: screening for TTR fibril disrupters. *FASEB J.* 17:803–809.
- Carell, K.W., and Lomas, D.A. (1997). Conformational disease. *Lancet* 350:134–138.

- Casanova, S., Donini, U., Zucchelli, P., Mazzucco, G., Monga, G., and Linke, R.P. (1992). Immunohistochemical distinction between amyloidosis and fibrillar glomerulopathy. *Am. J. Clin. Pathol.* 97:787–795.
- Castano, E.M., Prelli, F., Morelli, L., Avagnina, A., Kahn, B., and Frangione, B. (1997). Immunoglobulin lambda light chains are the precursors of urethral localized amyloidosis: a micromethod for extraction of amyloid. *Amyloid: J. Prot. Fold. Disord.* 4, 253–258.
- Cathcart, E.S., Skinner, M., and Cohen, A.S. (1971). Immunogenicity of amyloid. *Immunology* 20:945.
- Caughey, B.W., Dong, A., Bhat, K.S., Ernst, D., Hayes, S.F., and Caughey, W.S. (1991). Secondary structure analysis of the scrapie-associated protein PrP27-30 in water by infrared spectroscopy. *Biochemistry* 30:7672–7680.
- Chastonay, P., and Hurlimann, J. (1986). Characterization of different amyloids with immunological techniques. *Pathol. Res. Pract.* 181:657–663.
- Cohen, A.S. (1967). Amyloidosis. *N. Engl. J. Med.* 277:522–530, 574–583, 628–638.
- Cohen, A.S., and Calkins, E. (1959a). Electron microscopic observation on a fibrous component in amyloid of diverse origin. *Nature* 183:1202–1203.
- Cohen, A.S., Calkins, E., and Levene, C. (1959b). Studies on experimental amyloidosis. Analysis of histology and staining sections of casein-induced amyloidosis in rabbits. *Am. J. Pathol.* 35:971–989.
- Colbatzky, F., Brunnberg, L., Linke, R.P., Geisel, O., and Hermanns, W. (1991). AA-like amyloid deposits confined to arthritic joints in two dogs with rheumatoid arthritis. *J. Comp. Pathol.* 105:331–343.
- Cooper, J.H. (1969). An evaluation of current methods for the diagnostic histochemistry of amyloid. *J. Clin. Pathol.* 22:410–413.
- Cooper, J.H. (1974). Selective amyloid staining as a function of amyloid composition and structure. Histochemical analysis of the alkaline congo red, standardized toluidine blue and iodine methods. *Lab. Invest.* 31:232–238.
- Cooper, J.H. (1976). “Natural” green birefringence of amyloid. *Lett. Am. J. Clin. Pathol.* 66:1028–1230.
- Cooper, J.H. (1981). A histochemical construct of the amyloid fibril. In: Tribes, C.R., and Bacon, P.A. (eds.), *Proc. First Eur. Amyloid Res. Symp.* London: J. Wright & Sons Ltd., pp. 31–34.
- Cooper, J.H. (1991). A histochemical model of the amyloid fibril. In: Natvig, J.B., Forre, O., Husby, A., Husebekk, A., Skogen, B., Sletten, K., and Westermark, P. (eds), *Amyloid and amyloidosis*. Dordrecht: Kluwer Acad. Publ., pp. 515–518.
- Cornwell, G.G., III, Husby, G., Westermark, P., Natvig, J.B., Michaelson, T.E., and Skogen, B. (1977). Identification and characterization of different amyloid fibril proteins in tissue sections. *Scand. J. Immunol.* 6:1071–1080.
- Costa, P.P., Figueira, A.S., and Bravo, F.R. (1978). Amyloid fibril protein related to prealbumin in familial amyloidotic polyneuropathy. *Proc. Natl. Acad. Sci. USA* 75:4499–4503.
- Dalakas, M.C., and Cunningham, C. (1986). Characterization of amyloid deposits in biopsies of 15 patients with “sporadic” (non-familial or plasma-cell dyscrasic) amyloid polyneuropathy. *Acta Neuropathol.* 69:66–72.
- Dalakas, M.C., Fujihara, S., Askanas, V., Engel, W.K., and Glenner, W.G. (1984). Nature of amyloid deposits in hypernephroma. Immunocytochemical studies in two cases associated with amyloid polyneuropathy. *Am. J. Pathol.* 116: 447–454.
- De Carvalho, M., Linke, R.P., Domingo, F., Evangelista, T., Ducla-Soares, J.L., Nathrath, W.B.J., Azevedo-Coutinho, C., Lima, R., and Saraiva, M.J. (2004). Mutant fibrinogen A- α -chain associated with hereditary renal amyloidosis and peripheral neuropathy. *Amyloid J. Protein Fold. Disord.* 11:200–207.
- DeLellis, R.A., Glenner, G.G., and Ram, J.S. (1968). Histochemical observations on amyloid with reference to polarization microscopy. *J. Histochem. Cytochem.* 16:663–664.
- Demaimay, R., Chesebro, B., and Caughey, B. (2000). Inhibition of protease-resistant prion protein by trypan blue, sirius red and other congo red analogs. *Arch. Virol. Suppl.* 16:277–283.
- Dezutter, N.A., Landmann, W.J.M., Jager, P.L., de Groot, T.J., Dupont, P.J., Tooten, P.C.J., Zekarias, B., Gruys, E., and Verbruggen, A.M. (2001). Evaluation of ^{99m}Tc -MAMA-chrysamine G as an in vivo probe for amyloidosis. *Amyloid J. Protein Fold. Disord.* 8:202–214.
- Diezel, P.B., and Pfeleiderer, A. (1959). Histochemische und polarisationsoptische Untersuchungen am Amyloid. *Virchows Arch. Pathol. Anat.* 332:552–567.
- Dispenzieri, A., Kyle, R.A., Lacy, M.Q., Therneau, T.M., Larson, D.R., Plevak, M.F., Rajkumar, S.V., Fonseca, R., Greipp, P.R., Witzig, T.E., Lust, J.A., Zeldenrust, S.R. Snow, D.S., Hayman, S.R., Litzow, M.R., Gastineau, D.A., Tefferi, A., Inwards, D.J., Micallef, I.N., Ansell, S.M., Porrata, L.F., Elliott, M.A., and Gertz, M.A. (2004). Superior survival in primary systemic amyloidosis patients undergoing peripheral blood stem cell transplantation: a case–control study. *Blood* 103:3960–3963.
- Dithmar, S., Linke, R.P., Kolling, G., and Völcker, H.E. (2004). Ptosis from localized AL λ -amyloid deposits in the levator palpebrae muscle. *Ophthalmology* 111:1043–1047.
- Divry, P., and Florkin, M. (1927). Sur les propriétés optiques de l’amyloïde. *C.R. Soc. Biol.* 97:1808–1810.
- Dobson, C.M. (2003). Protein folding and misfolding. *Nature* 426(6968):884–890.

- Donini, U., and Linke R.P. (2004). Congo red fluorescence circumvents pitfalls in morphometric quantitation of amyloid in tissue sections. In: Grateau, G., Kyle, R.A., and Skinner, M. (eds.), *Amyloid and amyloidosis*. Boca Raton, FL: CRC Press, pp. 82–84.
- Donini, U., Casanova, S., Dal Bosco, F., and Linke R.P. (1984). Immunohistochemical typing of amyloid on hydroxyethyl-methacrylate-embedded renal biopsies. *Appl. Pathol.* 2:299–307.
- Donini, U., Casanova, S., Zucchelli, P., and Linke, R.P. (1989). Immunoelectron microscopic classification of amyloid on renal biopsies. *J. Histochem. Cytochem.* 37:1101–1106.
- Donini, U., Casanova, S., Zucchelli, P., Poli, C., Carboni, G., and Linke, R.P. (1991). Computer-aided immunohistochemical quantitation of amyloid in renal biopsies. In: Natvig, J.B., Forre, O., Husby, G., Husebekk, A., Skogen, B., Sletten, K., and Westermark, P. (eds.), *Amyloid and amyloidosis*. Dordrecht: Kluwer Acad. Publ., pp. 793–796.
- Eanes, E.D., and Glenner, G.G. (1968). X-ray diffraction studies on amyloid filaments. *J. Histochem. Cytochem.* 16:673–677.
- Edwards, R.A., and Woody, R.W. (1979). Spectroscopic studies of cibacron blue and congo red bound to dehydrogenases and kinases: evaluation of dyes as probes of the dinucleotide fold. *Biochemistry* 18:5197–5204.
- Ericzon, B.G., Suhr, O., Broome, U., Holmgren, G., Duraj, F., Eleborg, L., Wikstrom, L., Norden, G., Friman, S., and Groth, C.G. (1995). Liver transplant holds the progress of familial amyloidotic polyneuropathy. *Transplant. Proc.* 27:1233.
- Falbe, J., and Regitz, M. (1990). *Römp-Chemie Lexikon*, 9th ed. Stuttgart: Georg Thieme Verlag.
- Falk, R.H., and Skinner, M. (2000). The systemic amyloidoses: an overview. *Adv. Int. Med.* 45:107–137.
- Feurle, G.E., Linke, R.P., Kuhn, E., and Wagner, A. (1984). Clinical value of immunohistochemistry with AF-antibody in the diagnosis of familial amyloid neuropathy. *J. Neurol.* 231:237–243.
- Frenzel, H., Schwarzkopf, B., Kuhn, H., Lössle, B., Thormann, J., Hort, W., and Linke, R.P. (1986). Cardiac amyloid deposits in endomyocardial biopsies. Light microscopy, ultrastructural and immunohistochemical studies. *Am. J. Clin. Pathol.* 6:674–682.
- Fujihara, S. (1982). Differentiation of amyloid fibril proteins in tissue sections. *Acta Pathol. Jpn.* 32:771–782.
- Fujihara, S., and Glenner, G.G. (1981). Primary localized amyloidosis of the genitourinary tract: immunohistochemical study on eleven cases. *Lab. Invest.* 44:55–60.
- Gafni, J., and Sohar, E. (1960). Rectal biopsy for the diagnosis of amyloidosis. *Am. J. Med. Sci.* 240:332–336.
- Gallo, G.R., Feiner, H.D., Katz, L.A., Feldman, G.M., Correa, E.B., Chuba, J.V., and Buxbaum, J.N. (1980). Nodular glomerulopathy associated with nonamyloidotic kappa light chain deposits and excess immunoglobulin light chain synthesis. *Am. J. Pathol.* 99:621–644.
- Gallo, G.R., Feiner, H.D., Chuba, J.V., Beneck, D., Marion, P., and Cohen, D.H. (1986). Characterization of tissue amyloid by immunofluorescence microscopy. *Clin. Immunol. Immunopathol.* 39:479–490.
- Geisel, O., Stiglmaier-Herb, M.T., and Linke, R.P. (1990). Myeloma associated with immunoglobulin lambda-light chain derived amyloid in a dog. *Vet. Pathol.* 27:374–376.
- Glabe, C.G. (2004). Conformation-dependent antibodies target diseases of protein misfolding. *Trends Biochem. Sci.* 29:542–577.
- Glenner, G.G. (1980). Amyloid deposits and amyloidosis: The β -fibrilloses. *N. Engl. J. Med.* 302:1283–1292, 1333–1343.
- Glenner, G.G. (1981). The bases of the staining of amyloid fibers: their physico-chemical nature and the mechanism of their dye–substrate interaction. *Prog. Histochem. Cytochem.* 13:1–37.
- Glenner, G.G., Ein, D., Eanes, E.D., Bladen, H.A., Terry, W., and Page, D.L. (1971a). Creation of “amyloid” fibrils from Bence-Jones proteins in vitro. *Science* 174:712–714.
- Glenner, G.G., Terry, W., Harada, M., Isersky, C., and Page, D. (1971b). Amyloid fibril proteins: proof of homology with immunoglobulin light chains by sequence analysis. *Science* 172:1150–1151.
- Glenner, G.G., Eanes, E.D., Bladen, H.A., Linke, R.P., and Termine, E.C. (1974). β -Sheet fibrils. A comparison of native amyloid with synthetic protein fibrils. *J. Histochem. Cytochem.* 22:1141–1158.
- Godbersen, G.S., Leh, J.F., Hansmann, M.L., Rudert, H., and Linke, R.P. (1992). Organ-limited laryngeal amyloid deposits: clinical, morphological, and immunohistochemical results of five cases. *Ann. Otol. Rhinol. Laryngol.* 101:770–775.
- Gono, T., Matsuda, M., Shimojima, Y., Ishii, W., Koyama, J., Sakashita, K., Koike, K., Hoshii, Y., and Ikeda, S.I. (2004). VAD with or without subsequent high-dose melphalan followed by autologous stem cell support in AL amyloidosis: Japanese experience and criteria for patient selection. *Amyloid J. Protein Fold. Disord.* 11:245–256.
- Hawkins, P.N. (1994). Diagnosing and monitoring of amyloidosis. *Baillières Clin. Rheumatol.* 8:635–659.
- Hawkins, P.N., Lavender, J.P., and Pepys, M. (1990). Evaluation of systemic amyloidosis by scintigraphy with ^{125}I -labeled serum amyloid P component. *N. Engl. J. Med.* 324:508–513.
- Hazenbergh, B.P.C., Limburg, P.C., Bijzet, J., and vanRijswijk, M.H. (1999). A quantitative method for detecting deposits of amyloid A protein in aspirated fat tissue of patients with arthritis. *Ann. Rheum. Dis.* 58:96–102.

- Heller, H., Missmahl, H.P., Sohar, E., and Gafni, J. (1964). Amyloidosis: its differentiation into perireticulin and pericollagen types. *J. Pathol. Bact.* 88:15–34.
- Herlenius, G., Wilczek, H.E., Larsson, M., and Ericzon, B.G. (2004). Familial amyloidotic polyneuropathy world transplant registry. Ten years of international experience with liver transplantation for familial amyloidotic polyneuropathy. *Transplantation* 77:64–71.
- Highman, B. (1946). Improved method for demonstrating amyloid in paraffin sections. *Arch. Pathol.* 41:559–562.
- Holmgren, G., Ericzon, B.G., Groth, C.G., Stehen, L., Suhr, O., Andersen, O., Wallin, B.G., Seymour, A., Richardson, S., Hawkins, P.N., and Pepys, M.B. (1993). Clinical improvement and amyloid regression after liver transplantation in hereditary transthyretin amyloidosis. *Lancet* 341:1113–1116.
- Husby, G., and Natvig, J.B. (1972). Immunologic characterization of amyloid fibrils in tissue sections. *Clin. Exp. Immunol.* 11:357–366.
- Ikeda, S., Wong, C.W., Alsop, D., Landon, M., Kidd, M., and Glenner, G.G. (1987). Immunogold labelling of cerebrovascular and neuritic plaque amyloid fibrils in Alzheimer's disease with anti β -protein monoclonal antibodies. *Lab. Invest.* 60:113–122.
- Ishii, K., Klunk, W.E., Arawaka, S., Debnath, N.L., Furiya, Y., Sahara, N., Shoji, S., Tamaoka, A., Pettegrew, J.W., and Mori, H. (2002). Chrysamine G and its derivative reduce amyloid beta-induced neurotoxicity in mice. *Neurosci. Lett.* 333:5–8.
- Isobe, T., and Osserman, E.F. (1974). Patterns of amyloidosis and their association with plasma cell dyscrasias, monoclonal immunoglobulins and Bence-Jones proteins. *N. Engl. J. Med.* 290:473–477.
- Isobe, T., Matsushita, T., Minakata, T., Takahashi, M., Hoshii, Y., Ishiwa, T., and Uchino, F. (1996). Coexistence of AL $A\beta$ 2M amyloid in tissues of a patient with myeloma on hemodialysis. *Amyloid Int. J. Exp. Clin. Invest.* 3:41–43.
- Jarret, J.T., and Landsbury, P.T. (1993). Seeding “one-dimensional crystallization” of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie. *Cell* 73:1055.
- Jenne, D.E., Denzel, K., Blättinger, P., Winter, P., Obermaier, B., Linke, R.P., and Altland, K. (1996). A new isoleucine substitution of Val-20 in transthyretin selectively impairs dimer–dimer contact and causes systemic amyloidosis. *Proc. Natl. Acad. Sci. USA* 93:6302–6307.
- Kaplan, B., Yakar, S., Kumar, A., Pras, M., and Gallo, G. (1997). Immunochemical characterization of amyloid in diagnostic biopsy tissues. *Amyloid J. Protein Fold. Disord.* 4:80–86.
- Kaplan, B., Hrnčić, R., Murphy, C.L., Gallo, G., Weiss, D.T., and Solomon, A. (1999). Microextraction and purification techniques applicable to chemical characterisation of amyloid proteins in minute amounts of tissue, vol. 309. In: Wetzel, R. (ed.), *Methods in enzymology*. San Diego: Academic Press, pp. 67–81.
- Kaplan, B., Murphy, C.L., Ratner, V., Pras, M., Weiss, D.T., and Solomon, A. (2001). Micromethod to isolate and purify amyloid proteins for chemical characterization. *Amyloid J. Protein Fold. Disord.* 8:22–29.
- Kaplan, B., Martin, B.M., Livneh, A., Pras, M., and Gallo, G.R. (2004). Biochemical subtyping of amyloid in formalin-fixed tissue samples confirms and supplements immunohistologic data. *Am. J. Clin. Pathol.* 121:794–800.
- Kheterpal, I., Zhou, S., Cook, K.D., and Wetzel, R. (2000). Abeta amyloid fibrils possess a core structure highly resistant to hydrogen exchange. *Proc. Natl. Acad. Sci. USA* 97:13597–13601.
- Kheterpal, I., Lashuel, H.A., Hartley, D.M., Walz, T., Landsbury, P.T., Jr., and Wetzel, R. (2003). Abeta protofibrils possess a stable core structure resistant to hydrogen exchange. *Biochemistry* 42:14092–14098.
- Kisilevsky, R. (2000). Review: amyloidogenesis-unquestioned answers and unanswered questions. *J. Struct. Biol.* 130: 99–108.
- Kisilevsky, R., and Fraser, P.E. (1997). $A\beta$ amyloidogenesis: unique or variation on a systemic theme. *Crit. Rev. Biochem. Mol. Biol.* 32:361–404.
- Kitamoto, T., Tateishi, J., Tashima, T., Takeshita, I., Barry, R.A., DeArmond, S.J., and Prusiner, S.B. (1986). Amyloid plaques in Creutzfeldt-Jakob disease stain with prion protein antibodies. *Ann. Neurol.* 20:204–208.
- Kitamoto, T., Ogomori, K., Tateishi, J., and Prusiner, S.B. (1987). Formic acid pretreatment enhances immunostaining of cerebral and systemic amyloids. *Lab. Invest.* 57:230–236.
- Klunk, W.E., Debnath, M.L., Koros, A.M., and Pettegrew, J.W. (1998). Chrysamine-G, a lipophilic analogue of Congo red, inhibits A beta-induced toxicity in PC12 cells. *Life Sci.* 63:1807–1814.
- Klunk, W.E., Wang, Y., Holt, D.P., Huang, G.F., Debnath, M.L., Holt, D.P., and Mathis, C.A. (2001). Uncharged thioflavin T derivatives bind to amyloid-beta protein with highly affinity and readily enter the brain. *Life Sci.* 69:1471–1484.
- Klunk, W.E., Barskai, B.J., Mathis, C.A., Kajdasz, S.T., McLellan, M.E., Frosch, M.P., Debnath, M.L., Holt, D.P., Wang, Y., and Hyman, B.T. (2002). Imaging A beta plaques in living transgenic mice with multiphoton microscopy and methoxy X04, a systemically administered congo red derivative. *J. Neuropathol. Exp. Neurol.* 61:797–805.

- Klunk, W.E., Engler, H., Nordberg, A., Bacskaï, B.J., Wang, Y., Price, J.C., Bergstrom, M., Hyman, B.T., Langstrom, B., and Mathis, C.A. (2003a). Imaging the pathology of Alzheimer's disease: amyloid-imaging with positron emission tomography. *Neuroimaging Clin. N. Am.* 13:781–789.
- Klunk, W.E., Engler, H., Nordberg, A., Wang, Y., Blomqvist, G., Holt, D.P., Bergstrom, M., Savitcheva, I., Huang, G.F., Estrada, S., Aussen, B., Debnath, M.L., Barletta, J., Price, J.C., Sandell, J., Lopresti, B.J., Wall, A., Koivisto, P., Antoni, G., Mathis, C.A., and Langstrom, B. (2003b). Imaging brain amyloid in Alzheimer's disease with Pittsburgh Compound-B. *Ann. Neurol.* 55:306–319.
- Kyle, R.A. (2001). Amyloidosis: the last three centuries. *Amyloid J. Protein Fold. Disord.* 8(Suppl. 2):7–8.
- Kyle, R.A., and Gertz, M.A. (1995). Primary systemic amyloidosis: clinical and laboratory features in 474 cases. *Semin. Hematol.* 32:45–59.
- Kyle, R.A., Gertz, M.A., and Linke, R.P. (1992). Amyloid localized to tendosynovium at carpal tunnel release. Immunohistochemical identification of amyloid type. *Am. J. Clin. Pathol.* 97:250–253.
- Lachmann, H.J., Chir, B., Booth, D.R., Booth, S.E., Bybee, A., Gilbertson, J.A., Gillmore, J.D., Pepys, M.B., and Hawkins, P.N. (2002). Misdiagnosis of hereditary amyloidosis as AL (primary) amyloidosis. *N. Engl. J. Med.* 346:1786–1791.
- Lachmann, H.J., Gallimore, R., Gillmore, J.D., Carr-Smith, H.D., Bradwell, A.R., Pepys, M.B., and Hawkins, P.N. (2003). Outcome in systemic AL amyloidosis in relation to changes in concentration of circulating free immunoglobulin light chains following chemotherapy. *British Journal of Haematology* 122:78–84.
- Ladewig, P. (1945). Double-refringence of the amyloid-congo-red-complex. *Nature* 156:81–82.
- Lansbury, P.T. (1992). In pursuit of the molecular structure of amyloid plaque—new technology provides unexpected and critical information. *Biochemistry* 31:6865–6870.
- Layfield, R., Bailey, K., Dineen, R., Mchrotra, P., Lowe, J., Allibone, R., Mayer, R.J., and Landon, M. (1997). Application of formalin fixation to the purification of amyloid proteins. *Anal. Biochem.* 253:142–144.
- Layfield, R., Bailey, K., Lowe, J., Allibone, R., Mayer, R.J., and Landon, M. (1996). Extraction and protein sequencing of immunoglobulin light chain from formalin-fixed cerebrovascular amyloid deposits. *J. Pathol.* 180:455–459.
- Levo, Y., Livni, N., and Laufer, A. (1980). Immuno-histological diagnosis and classification of amyloidosis. In: Glenner, G.C., Costa, P.P., and Freitas, A.F. (eds.), *Amyloid and amyloidosis*. Amsterdam: Excerpta Medica, pp. 35–37.
- Li, K., Kyle, R.A., and Dyck, P.J. (1992). Immunohistochemical characterization of amyloid proteins in sural nerves and clinical association in amyloid neuropathy. *Am. J. Pathol.* 141:217–226.
- Linke, R.P. (1982). Immunohistochemical identification and cross reactions of amyloid fibril protein in senile heart and in familial polyneuropathy. Lack of reactivity with cerebral amyloid in Alzheimer's disease. *Clin. Neuropathol.* 1:172–182.
- Linke, R.P. (1984). Monoclonal antibodies against amyloid fibril protein AA. Production, specificity and use for immunohistochemical localization and classification of AA-type amyloidosis. *J. Histochem. Cytochem.* 32:322–328.
- Linke, R.P. (1985). Immunochemical typing of amyloid deposits after microextraction from biopsies. *Appl. Pathol.* 3:18–28.
- Linke, R.P. (1987). The amyloidoses. Pathogenetic accurate classification and amyloid-type specific therapy. *Nieren. Hochdruckkr.* 16:145–153.
- Linke, R.P. (2000). Highly sensitive diagnosis of amyloid and various amyloid syndromes using congo red fluorescence. *Virchows Arch. Pathol. Anat.* 436:439–448.
- Linke, R.P., and Nathrath, W.B.J. (1980). Classification of amyloidoses at biopsy by the immunoperoxidase method. *Münchener. Med. Wochenschrift* 49:1772–1777.
- Linke, R.P., Zucker-Franklin, D., and Franklin, E.C. (1973). Morphologic, chemical and immunological studies of amyloid-like fibrils formed from Bence Jones proteins by proteolysis. *J. Immunol.* 111:10–23.
- Linke, R.P., Heilmann, K.L., Nathrath, W.B.J., and Eulitz, M. (1983a). Identification of amyloid A protein in a sporadic Muckle-Wells-syndrome. N-terminal amino acid sequence analysis after isolation from formalin-fixed tissue. *Lab. Invest.* 48:698–704.
- Linke, R.P., Nathrath, W.B.J., and Wilson, P.D. (1983b). Immuno-electron microscopic identification and classification of amyloid in tissue sections by the postembedding protein-A gold method. *Ultrastruct. Pathol.* 4:1–7.
- Linke, R.P., Nathrath, W.B.J., and Eulitz, M. (1984). Classification of amyloid syndromes from tissue sections using antibodies against various amyloid fibril proteins: report of 142 cases (Abstr.). 4th Internat. Symp. *Amyloid and Amyloidosis*, Harriman, NY.
- Linke, R.P., Kunert, U., Lobeck, H., Hampl, H., and Eulitz, M. (1988). Chemical analysis of beta2-microglobulin-derived amyloid in patients on long-term hemodialysis. In: Isobe, T., Araki, S., Uchino, F., Kito, S., and Tsubura, E. (eds), *Amyloid and amyloidosis*. New York: Plenum Press, pp. 611–616.

- Linke, R.P., Huhn, D., Casanova, S., and Donini, U. (1989). Immunoelectron microscopic identification of human AA-type amyloid: exploration of various monoclonal AA-antibodies, methods of fixation, embedding and other parameters for the protein-A gold method. *Lab. Invest.* 61:691–697.
- Linke, R.P., Gärtner, H., and Michels, H. (1995). High-sensitivity diagnosis of AA amyloidosis using Congo red and immunohistochemistry detects missed amyloid deposits. *J. Histochem. Cytochem.* 43:863–869.
- Linke, R.P., Altland, K., Ernst, J., Gerhard, L., Michels, H., Saeger, W., and Willig, F. (1998). Practical advice in diagnosis and treatment of systemic amyloidosis. *Deutsch. Ärztebl.* 95:2626–2636 [idem: (1999) 96A-861–A-862].
- Linke, R.P., Joswig, R., Murphy, C.L., Wang, S., Zhou, H., Gross, U., Röcken, C.B., Nathrath, W.B.J., Westermarck, P., Weiss, D., and Solomon, A. (2004). Semenogelin I is the amyloidogenic protein in senile seminal vesicles. In: Grateau, G., Kyle, R.A., and Skinner, M. (eds.), Boca Raton, FL: CRC Press, pp. 471–473.
- Loienzo, A., and Yankner, B.A. (1994). β -amyloid neurotoxicity requires fibril formation and is inhibited by Congo red. *Proc. Natl. Acad. Sci. USA* 91: 12243–12247.
- Lubarsch, O. (1929). Zur Kenntnis ungewöhnlicher Amyloidablagerungen. *Virchows Arch. Pathol. Anat.* 271:367.
- Majzoub, M., Breuer, W., Platz, S.L., Linke, R.P., and Hermanns, W. (2003). Histopathologic and immunophenotypic characterization of extramedullary plasmacytomas in nine cats. *Vet. Pathol.* 40:249–253.
- McCubbin, W.D., Kay, C.M., Narindrasorasak, S., and Kisilevsky, R. (1988). Circular dichroism and fluorescence studies on two murine serum amyloid A proteins. *Biochem. J.* 256:775–783.
- Meloan, S.N., and Puchtler, H. (1978). Demonstration of amyloid with Mesitol WLS-Congo red: application of a textile auxiliary to histochemistry. *Histochemistry* 58:163–166.
- Merlini, G., and Belotti, V. (2003). Molecular mechanisms of amyloidosis. *N. Engl. J. Med.* 349:583–596.
- Merlini, G., and Westermarck, P. (2004). The systemic amyloidoses: clearer understanding of the molecular mechanisms offer hope for more effective therapies. *J. Intern. Med.* 255:159–178.
- Merlini, G., Anesi, E., Garini, P., Perfetti, V., Obici, L., Ascari, E., Lechuga, M.H., Capri, G., and Gianni, L. (1999). Treatment of AL amyloidosis with 4'-iodo-4'-deoxydoxorubicin: an update. *Blood* 93:1112–1113.
- Michels, H., and Linke, R.P. (1998). Clinical benefit of diagnosing incipient AA-amyloidosis in pediatric rheumatic diseases as estimated from a retrospective study. *Amyloid J. Protein Fold. Disord.* 5:200–207.
- Michels, H., Donini, U., and Linke, R.P. (1994). Resolution of AA-amyloidosis in three patients with juvenile rheumatoid arthritis (JRA) as shown with computer-aided quantitation of amyloid. In: Kisilevsky, R., Benson, M.D., Frangione, B., Gauldie, J., Muckle, T.J., and Young, I.D. (eds.), *Amyloid and amyloidosis*. New York: Parthenon Publ., pp. 694–696.
- Missmahl, H.P. (1950). Histochemische Versuche an der Amyloidsubstanz. *Virchows Arch. Pathol. Anat.* 318:518–533.
- Missmahl, H.P., and Hartwig, M. (1953). Polarisationsoptische Untersuchungen an der Amyloidsubstanz. *Virchows Arch. Pathol. Anat.* 324:489–508.
- Miura, T., Yamamiya, C., Sasaki, M., Suzuki, K., and Takeuchi, H. (2002). Binding mode of congo red to Alzheimer's amyloid beta-peptide studied by UV Raman spectroscopy. *J. Raman. Spectrosc.* 33:530–535.
- Murphy, C.L., Eulitz, M., Hrnec, R., Sletten, K., Westermarck, P., Williams, T., Macy, S.D., Wooliver, C., Wall, J., Weiss, D.T., and Solomon, A. (2001). Chemical typing of amyloid protein contained in formalin-fixed paraffin-embedded biopsy specimens. *Am. J. Clin. Pathol.* 116:135–142.
- Newland, J.R., Linke, R.P., and Lennert, K. (1986). Amyloid deposits in lymph nodes. A morphologic and immunohistochemical study. *Hum. Pathol.* 17:1245–1249.
- Ofri, R., Nyska, A., Linke, R.P., Shtrasburg, S., Livneh, A., and Gal, R. (1997). Systemic amyloidosis in a cheetah (*Acinonyx jubatus*). *Amyloid Int. J. Exp. Clin. Invest.* 4:98.
- O'Nuallain, B., and Wetzel, R. (2002). Conformational Abs recognizing a generic amyloid fibril epitope. *Proc. Natl. Acad. Sci. USA* 99:1485–1490.
- Orfila, C., Giraud, P., Modesto, A., and Suc, J.M. (1986). Abdominal fat tissue aspirate in human amyloidosis: light, electron and immunofluorescence microscopic studies. *Hum. Pathol.* 17:366–369.
- Pepys, M.B. (2001). Pathogenesis, diagnosis and treatment of systemic amyloidosis. *Philos. Trans. R. Soc. Lond. Series B: Biol. Sci.* 356:203–210 [discussion 210–211].
- Pepys, M.B., Booth, D.R., Hutchinson, W.L., Gallimore, J.R., Collins, P.M., and Hohenester, E. (1997). Amyloid P component: a critical review. *Amyloid Int. J. Exp. Clin. Invest.* 4:274–295.
- Perz, J., Schönland, S.O., Hundemer, M., Linke, R.P., Zeier, M., Ho, A.D., and Goldschmidt, H. (2004). High-dose melphalan with autologous stem cell transplantation after VAD induction chemotherapy for treatment of AL amyloidosis: a single centre prospective phase II study. *Br. J. Haematol.* 127:543–551.
- Picken, M.M., Frangione, B., Barlogie, B., Luna, M., and Gallo, G. (1989). Light chain deposition disease derived from the κ -chain subgroup. Biochemical characterization. *Am. J. Pathol.* 134:749–754.

- Platz, S.J., Breuer, W., Geisel, O., Linke, R.P., and Hermanns, W. (1997). Identification of λ light chain amyloid in eight canine and two feline extramedullary plasmacytomas. *J. Comp. Pathol.* 16:45–54.
- Pras, M., and Schubert, M. (1969). Metachromatic properties of amyloid in solution. *J. Histochem. Cytochem.* 17:258–265.
- Pras, M., Schubert, M., Zucker-Franklin, D., Rimon, A., and Franklin, E.C. (1968). The characterization of soluble amyloid prepared in water. *J. Clin. Invest.* 47:924–933.
- Pras, M., Nevo, Z., Schubert, M., Rotman, J., and Matalon, R. (1971). The significance of mucopolysaccharides in amyloid. *J. Histochem. Cytochem.* 19:443.
- Puchtler, H., and Sweat, F. (1965). Congo red as a stain for fluorescence microscopy of amyloid. *J. Histochem. Cytochem.* 13:693–694.
- Puchtler, H., and Sweat, F. (1966). A review of early concepts of amyloid in context with contemporary chemical literature from 1839 to 1959. *J. Histochem. Cytochem.* 14:123.
- Puchtler, H., Sweat, F., and Levine, M. (1962). On the binding of congo red by amyloid. *J. Histochem. Cytochem.* 10:355–364.
- Puchtler, H., Sweat, F., and Kuhns, J.G. (1964). On the binding of direct cotton dyes by amyloid. *J. Histochem. Cytochem.* 12:900–907.
- Puchtler, H., Waldrop, F.S., and Meloan, S.N. (1985). A review of light polarization and fluorescence microscopic methods for amyloid. *Appl. Pathol.* 3:5–17.
- Puille, M., Altland, K., Linke, R.P., Stehen-Müller, M.K., Klett, R., Steiner, D., and Bauer, R. (2002). Tc-99m-DPD scintigraphy in transthyretin-related familial amyloid polyneuropathy (FAP). *Eur. J. Nucl. Med.* 29:376–379.
- Reiman, H.A., Koucky, R.F., and Eklund, C.M. (1935). Primary amyloidosis limited to tissue of mesodermal origin. *Am. J. Pathol.* 11:977–988.
- Röcken, C., Saeger, W., and Linke, R.P. (1996a). Portal amyloid: a novel amyloid deposit in gastrointestinal veins. *Arch. Pathol. Lab. Med.* 120:1044–1051.
- Röcken, C., Schwotzer, E.B., and Linke, R.P. (1996b). The classification of amyloid deposits in clinicopathologic practice. *Histopathology* 29:325–335.
- Romhányi, G. (1943). Über die submikroskopische Struktur des Amyloids (Abstr.). *Zentralbl. Allg. Pathol.* 80:411.
- Romhányi, G. (1949). Über die submikroskopische Struktur des Amyloids. *Pathol. Bakt.* 12:253–262.
- Romhányi, G. (1959). Zur Frage der submikroskopischen Struktur des Amyloids. *Zentr. Allg. Pathol. Anat.* 95:130–138.
- Romhányi, G. (1971). Selective differentiation between amyloid and connective tissue structures based on the collagen-specific topo-optical staining reaction with congo red. *Virchows Arch. Pathol. Anat.* 354:209–222.
- Romhányi, G. (1972). Differences in ultrastructural organization of amyloid as revealed by sensitivity or resistance to induced proteolysis. *Virchows Arch. Pathol. Anat.* 357:29–52.
- Roterman, I., Krui, M., Nowak, M., Konieczny, L., Rybarska, J., Stopa, B., Piekarska, B., and Zemanek, G. (2001). Why Congo red binding is specific for amyloid proteins—model studies and a computer analysis approach. *Med. Sci. Monitor* 7:771–784.
- Royston, M.C., Kodical, N.S., Mann, D.M., Groom, K., Landon, M., and Roberts, G.W. (1994). Quantitative analysis of beta-amyloid deposition in Downs Syndrome using computerized image analysis. *Neurodegeneration* 3:43–51.
- Rudyk, H., Knaggs, M.H., Vasiljevic, S., Hope, J., Birkett, C.R., and Gilbert, I.H. (2003). Synthesis and evaluation of analogues of Congo red as potential compounds against transmissible spongiform encephalopathies. *Eur. J. Med. Chem.* 38:567–579.
- Staunton, H., Devan, P., Kale, R., Linke, R.P., and Kelly, P. (1987). Hereditary amyloid polyneuropathy in North West Ireland. *Brain* 110:1231–1246.
- Schröder, R., and Linke, R.P. (1999). Cerebrovascular involvement in systemic AA- und AL-amyloidosis: a clear hematogenic pattern. *Virchows Arch. Pathol. Anat.* 434:551–560.
- Schröder, R., Linke, R.P., Voges, J., Heindel, W., and Sturm, V. (1995). Intracerebral AL-amyloidoma diagnosed by stereotactic biopsy. *Clin. Neuropathol.* 14:347–350.
- Schröder, R., Nennesmo, I., and Linke, R.P. (2000). Amyloid in multiple sclerosis lesions is clearly of AL λ type. *Acta Neuropathol.* 100:709–711.
- Schulz, C., Brüggmann, Boer, M., Brandt, H.P., Pohlenz, J., and Linke, R.P. (1998). Generalized AA-amyloidosis in Siberian tigers (*Panthera tigris altaica*) with predominant renal medullary amyloid deposition. *Vet. Pathol.* 35:70–74.
- Seldin, D.C., Anderson, J.J., Sancharawala, V., Malek, K., Wright, D.G., Quillen, K., Finn, K.T., Berk, J.L., Dember, L.M., Falk, R.H., and Skinner, M. (2004). Improvement in quality of life of patients with AL amyloidosis treated with high-dose melphalan and autologous stem cell transplantation. *Blood* 104:1888–1893.

- Selikoff, I.J., and Robitzek, E.H. (1947). Gingival biopsy for the diagnosis of generalized amyloidosis. *Am. J. Pathol.* 23:1099.
- Sellarajah, S., Lekishvili, T., Bowring, C., Thompsett, A.R., Rudyk, H., Birkett, C.R., Brown, D.R., and Gilbert, I.H. (2004). Synthesis of analogues of Congo red and evaluation of their anti-prion activity. *J. Med. Chem.* 47: 5515–5534.
- Serpell, L.C., Fraser, P.E., and Sunde, M. (1999). X-ray fiber diffraction of amyloid fibrils. In: Wetzel, R. (ed.), *Methods in enzymology*, vol. 309. San Diego: Academic Press, pp. 526–536.
- Shmueli, U., Gafni, J., Sohar, E., and Ashkenazi, Y. (1969). An X-ray study of amyloid. *J. Mol. Biol.* 41:309–311.
- Shtrasburg, S., Pras, M., Langevitch, P., and Gal, R. (1982). Demonstration of AA-protein in formalin-fixed, paraffin-embedded tissues. *Am. J. Pathol.* 106:141–144.
- Skinner, M., Sanchawala, V., Seldin, D.C., Dember, L.M., Falk, R.H., Berk, J.L., Anderson, J.J., O'Hara, C., Finn, K. T., Libbey, C.A., Wiesman, J., Quillen, K., Swan, N., and Wright, D.G. (2004). High-dose melphalan and autologous stem cell transplantation in patients with AL amyloidosis: an 8-year study. *Ann. Intern. Med.* 140:85–93.
- Skowronek, M., Roterman, I., Konieczny, L., Stopa, B., Rybarska, J., Piekarka, B., Górecki, A., and Król, M. (2000). The conformational characteristics of congo red, evans blue and trypan blue. *Comput. Chem.* 24:429–450.
- Snow, A.D., Kisilevsky, R., Stephens, C., and Anastasiades, T. (1987). Characterization of tissue and plasma glycosaminoglycans during experimental AA amyloidosis and acute inflammation. Quantitative and qualitative analysis. *Lab. Invest.* 56:665–675.
- Solomon, A., Murphy, C.L., Weaver, K., Weiss, D.T., Hrnec, R., Eulitz, M., Donnell, R.L., Sletten, K., Westermark, G., and Westermark, P. (2003a). Calcifying epithelial odontogenic (Pindborg) tumor-associated amyloid consists of a novel human protein. *J. Lab. Clin. Med.* 142:348–355.
- Solomon, A., Weiss, D.T., and Wall, J.S. (2003b). Immunotherapy in systemic primary (AL) amyloidosis using amyloid-reactive monoclonal antibodies. Review. *Cancer Biother. Radiopharmaceut.* 18:853–860.
- Spiro, D. (1959). The structural basis of proteinuria in man. Electron microscopic studies of renal biopsy specimens from patients with lipid nephrosis, amyloidosis and subacute and chronic glomerulonephritis. *Am. J. Pathol.* 35:47.
- Steensma, D.P. (2001). "Congo" red. Out of Africa. *Arch. Pathol. Lab. Med.* 125:250–252.
- Stein, K., Störkel, S., Linke, R.P., and Goebel, H.H. (1987). Chemical heterogeneity of amyloid in the carpal tunnel syndrome. *Virchows Arch. Path. Anat.* 412:37–45.
- Stiller, D., and Katenkamp, D. (1970). Untersuchungen zum fluoreszenzoptischen Nachweis von Amyloid mit Thioflavin S. *Zentralbl. Allg. Pathol.* 113:451–465.
- Stiller, D., Katenkamp, D., and Thoss, K. (1972). Der Färbemechanismus des Thioflavin T unter besonderer Berücksichtigung der Amyloiddarstellung. *Acta Histochem.* 42:234–245.
- Störkel, S., and Sturer, A. (1989). Combined amyloidosis of the AA and AB type following chronic hemodialysis. *Pathologie* 10:107–130.
- Strege, R.J., Saeger, W., and Linke, R.P. (1998). Immunohistochemical classification of systemic amyloidoses: Report on 45 consecutive autopsy cases. *Virchows Arch. Pathol. Anat.* 433:19–22.
- Strittmatter, W.J., and Roses, A.D. (1996). Apolipoprotein E and Alzheimer's disease. *Annu. Rev. Neurosci.* 19:53–77.
- Suhr, O.B., Holmgren, G., Steen, L., Wikstrom, L., Norden, G., Friman, S., Duraj, F.F., Groth, C.G., and Ericzon, B.G. (1995). Liver transplantation in familial amyloidotic polyneuropathy—follow-up of the first 20 Swedish patients. *Transplantation* 60:933–938.
- Sunde, M., and Blake, C.C. (1998). From the globular to the fibrous state: protein structure and structural conversion in amyloid formation. *Q. Rev. Biophys.* 31(1):1–39.
- Sweat, F., and Puchtler, H. (1965). Demonstration of amyloid with direct cotton dyes. Experiences with a new method for the selective staining of amyloid by Sirius Red F3B4 and Sirius Supra Scarlet GG-CF. *Arch. Pathol.* 80: 613–620.
- Takei, Y., Hattori, T., and Ikeda, S. (2001). Comparison of sural nerve findings before and after liver transplantation in FAP patients. *Amyloid J. Protein Fold. Disord.* 8(Suppl. 2):115.
- Tennent, G.A. (1999). Isolation and characterization of amyloid fibrils from tissue. In: Wetzel, R. (ed.), *Methods in enzymology*, vol. 309. San Diego: Academic Press, pp. 26–47.
- Termine, J., Eanes, E.D., Ein, D., and Glenner, G.G. (1972). Infrared spectroscopy of human amyloid fibrils and immunoglobulin proteins. *Biopolymers* 11:1103–1113.
- Townsend, R., Kumosinski, T.F., Timasheff, S.N., Fasman, G.D., and Davidson, B. (1966). The circular dichroism of the β -structure of poly-L-lysine. *Biochem. Biophys. Res. Commun.* 23:163–169.
- Turner, W.J., Cleveland, A.B., and Gorevic, P.D. (1983). A novel method for the typing of amyloid in small biopsy specimens. Western blot analysis of two-dimensional gels. *Clin. Res.* 31:355.

- Van de Kaa, C., Hol, P.R., Huber, J., Linke, R.P., Kooiker, C.J., and Gruys, E. (1986). Diagnosis of type of amyloid in paraffin wax embedded tissue sections using antisera against human and nimal amyloid proteins. *Virchows Arch. Pathol. Anat.* 408:649–664.
- Van Rijswijk, M.H., and van Heusden, C.W.G.J. (1979). The potassium permanganate method: a reliable method for differentiating amyloid AA from other forms of amyloid in routine laboratory practice. *Am. J. Pathol.* 97:43–58.
- Vassar, P.S., and Culling, C.F. (1959). Fluorescent stains, with special reference to amyloid and connective tissues. *Arch. Pathol.* 68:487–498.
- Virchow, R. (1854). Über eine im Gehirn und Rückenmark des Menschen aufgefundene Substanz mit der chemischen Reaktion der Cellulose. *Virchows Arch. Pathol. Anat.* 6:135–138.
- Waldrop, F.S., Puchtler, H., and Valentine, L.S. (1973). Fluorescence microscopy of amyloid using mix illumination. *Arch. Pathol.* 95:37–41.
- Walker, L.C., and LeVine, H. (2000). The cerebral proteopathies: neurodegenerative disorders of protein conformation and assembly. *Mol. Neurobiol.* 21:83–95.
- Wälti, O. (1945). Beitrag zur Kenntnis des Aufbaus der Zellulose. *Schweiz. Arch. Angew. Wissensch. Technik* 11:129–138, 181–189, 241–246.
- Wang, Y., Klunk, W.E., Debnath, M.L., Huang, G.F., Shao, L., and Mathis, C.A. (2004). Development of a PET/SPECT agent for amyloid imaging in Alzheimer's disease. *J. Mol. Neurosci.* 24:55–62.
- Westermarck, G., Johnson, K.H., and Westermarck, P. (1999). Staining methods for identification of amyloid in tissue. In: Wetzel, R. (ed.), *Methods in enzymology*, vol. 309. San Diego: Academic Press, pp. 3–25.
- Westermarck, P., and Stenkvist, B. (1973). A new method for the diagnosis of systemic amyloidosis. *Arch. Intern. Med.* 132:522–523.
- Westermarck, P., Wernstedt, C., Wilander, E., Hayden D.W., O'Brien, T.D., and Johnson, K.H. (1987). Amyloid fibrils in human insulinoma and islets of Langerhans of the diabetic cat are derived from a neuropeptide-like protein also present in normal islet cells. *Proc. Natl. Acad. Sci. USA* 84:3881–3885.
- Westermarck, P., Benson, M.D., Juul, J., and Sletten, K. (1989). Use of subcutaneous abdominal fat biopsy specimen for detailed typing of amyloid fibril protein-AL by amino acid esquence analysis. *J. Clin. Pathol.* 42:817–819.
- Westermarck, P., Benson, M.D., Buxbaum, J.N., Cohen, A.S., Frangione, B., Ikeda, S., Masters, C.L., Merlini, G., Saraiva, M.J., and Sipe, D.J. (2002). Amyloid fibril protein nomenclature. *Amyloid J. Protein Fold. Disord.* 9:197–200.
- Wetzel, R. (2002). Ideas of order for amyloid fibril structure. Review. *Structure* 10:1031–1036.
- Wiesehan, K., Buder, K., Linke, R.P., Patt, S., Stoldt, M., Unger, E., Schmitt, B., Bucci, E., and Willibold, D. (2003). Selection of D-amino-acid peptides that bind to Alzheimer's disease amyloid peptide A β_{1-42} by mirror image phage display. *Chem. Biol. Chem.* 4:748–753.
- Wolman, M. (1971). Amyloid, its nature and molecular structure: comparison of a new toluidine blue polarized light method with tradit. procedures. *Lab. Invest.* 25:104–110.
- Wolman, M., and Bubis, J.J. (1965). The cause of green polarization color of amyloid stained with Congo red. *Histochemie* 4:351–356.
- Wright, J.R., Calkins, E., and Humphrey, R.L. (1977). Potassium permanganate reaction in amyloidosis: a histologic method to assist in differentiating forms of this disease. *Lab. Invest.* 36:274–281.
- Yazaki, M., Liepnieks, J.J., Callaghan, J., Connolly, C.E., and Benson, M.D. (2004). Chemical characterization of a lamda I amyloid protein isolated from formalin-fixed and paraffin-embedded tissue sections. *Amyloid J. Protein Fold. Disord.* 11:50–55.
- Zemer, D., Pras, M., Sohar, E., Modan, M., Cabili, S., and Gafni, J. (1986). Colchicine in the prevention and treatment of the amyloidosis of familial mediterranean fever. *N. Engl. J. Med.* 314:1001–1005.
- Zeier, M., Perz, J., Linke, R.P., Donini, U., Waldherr, R., Andrassy, K., Ho, A.D., and Goldschmidt, H. (2003). No regression of renal AL-amyloid in monoclonal gammopathy after successful autologous blood stem cell transplantation and significant clinical improvement. *Nephrol. Dial. Transplant.* 18:2644–2647.
- Zschesche, W., and Linke, R.P. (1989). Immunohistochemical characterization of spontaneous amyloidosis in captive birds as AA-type, using monoclonal and polyclonal anti-AA antibodies against mammalian amyloid-A. *Acta Histochem.* 86:45–50.

Immunohistological Study of Experimental Murine AA Amyloidosis

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1. Abstract

The localization of amyloid fibril components and the cells related to the formation and resorption of the fibrils are still controversial. In this study we undertook a time-kinetic study to analyze the process of amyloid fibril deposition in the spleen of AA amyloidosis animal model immunohistochemically and ultrastructurally. Murine amyloid A (AA) amyloidosis was induced by the emulsion injection composed of Freund's complete adjuvant and *Mycobacterium butyricum*. Serum amyloid A (SAA) level was the highest at 3 days after the induction and gradually decreased. The amyloid deposition was first detected in extracellular spaces in the marginal zone of the spleen at 7 days after induction. The F4/80 positive red pulp macrophages increased in number after the induction and accumulated near the amyloid deposition areas. Amyloid P component (APC) and chondroitin sulfate proteoglycan (CSPG), which are composed of amyloid fibril, were detected in the cytoplasm of F4/80 positive red pulp macrophages and ER-TR9-positive marginal zone macrophages, respectively, then localized in the amyloid deposition areas. APC was also localized in CSPG-positive and F4/80-negative cells, which might be fibroblasts at 3 days. Ultrastructural examination indicated that macrophages in the marginal zone contained lysosome-derived fibrillar structures of amyloid, and that fibroblasts extended amyloid fibrils into the extracellular area in the marginal zone. These results suggested the close association of APC-positive/ER-TR9-positive macrophages and APC-positive/CSPG-positive fibroblasts with the formation of amyloid fibrils and F4/80-positive macrophages with the resorption of the fibrils.

2. Introduction

Amyloidosis is associated with a wide range of medical disorders including cancer, rheumatoid arthritis, Alzheimer's disease, chronic renal dialysis, familial amyloid polyneuropathy, and diabetes, but amyloidosis can also occur in the absence of a malady (Sipe, 1994). Amyloidosis is characterized by deposition of amyloid fibrils in the extracellular spaces of organs (Glenner *et al.*, 1973; Sipe, 1994; Inoue *et al.*, 1997, 1998a, 1999). Amyloid fibril deposition can be systemic, involving multiple organs or a single organ such as the pancreas in diabetes or the brain in Alzheimer's disease. Amyloid A (AA) amyloidosis is a systemic amyloidosis, and has been considered to be an inconsequential pathologic entity that causes significant clinical problems (Sipe, 1994). AA amyloidosis is also characterized by the deposition of AA amyloid fibrils in the extracellular matrix in various organs. The amyloid deposition detected by staining with Congo red shows a red-green color under polarized light

(Kuroiwa *et al.*, 2003). Biochemical research in AA amyloidosis has shown that the AA amyloid fibrils are derived from proteolytic cleavage of serum amyloid A (SAA), the precursor protein of the AA amyloid (Glenner *et al.*, 1973).

Animal models of AA amyloidosis have been used to clarify the amyloid pathogenesis (Takahashi *et al.*, 1985; Uchino *et al.*, 1985; Takahashi *et al.*, 1989; Du and Ali-Khan, 1990; Snow *et al.*, 1991; Lyon *et al.*, 1993; Inoue and Kisilevsky, 1996; Inoue *et al.*, 1998b, 2002; Togashi *et al.*, 1997; Kuroiwa *et al.*, 2003). Studies of animal models have shown an increase of SAA levels and the accumulation of amyloid P components (APC) and proteoglycans in the spleen, liver, and kidney. In the spleen, the AA amyloid fibril depositions have been found in the marginal zone. High-resolution ultrastructural and compositional analysis of the AA amyloid fibrils have revealed that the core is covered by heparan sulfate proteoglycan (HSPG) with a loose assembly of 1 nm-wide AA protein filaments (Inoue and Kisilevsky, 1996; Inoue *et al.*, 1998b, 2002). The core is a microfibril-like structure in which APC is enclosed in a tight helical structure by chondroitin sulfate proteoglycan (CSPG). SAA and APC are synthesized in hepatocytes (Benson and Kleiner, 1980; Tatsuta *et al.*, 1983) and fibroblasts also produce SAA (Linder *et al.*, 1976), but amyloid fibrils are deposited in the splenic marginal zone. Some studies indicate that reticuloendothelial cells might play an important role in the formation of amyloid fibrils (Du and Ali-Khan, 1990; Shirahama and Cohen, 1973, 1975; Chronopoulos *et al.*, 1994). Other research indicates that the intracytoplasmic amyloid fibrils in macrophages are derived from phagocytosis or the synthesis of new amyloids by macrophages *in situ* (Takahashi *et al.*, 1989). These results strongly suggest that both fibroblasts and macrophages might play important roles in the formation and resorption of fibrils, although it is still controversial which cells are directly related to this fibril formation and resorption. Furthermore, the localization of amyloid fibril components during amyloidogenesis is still not clear.

The purpose of this chapter is to define the cells concerned with the formation and resorption of amyloid fibrils in the spleen under experimental murine AA amyloidosis by the immunohistochemical detection of amyloid components and cells.

3. Amyloid Induction

Amyloid A amyloidosis can be induced in mice by the injection of casein, lipopolysaccharide, or emulsion of heat-killed *Mycobacterium butyricum* (*M. butyricum*) in Freund's complete adjuvant (FCA) (Takahashi *et al.*, 1985, 1989; Uchino *et al.*, 1985; Lyon *et al.*, 1993; Togashi *et al.*, 1997; Kuroiwa *et al.*, 2003). AA amyloid deposition occurs primarily in the spleen, liver, and kidney within 7–10 days after injection. With the addition of amyloid-enhancing factor (AEF) and an inflammatory stimulus such as silver nitrate (Snow *et al.*, 1991; Inoue and Kisilevsky, 1996; Inoue *et al.*, 1998b, 2002), AA amyloid deposition occurs within 24–48 hours in the spleen, liver, and kidney. Although the source of AEF is the spleen or liver from an amyloidotic animal, AEF has not yet been well defined except for its dramatic biologic effect.

In this chapter, AA amyloidosis was induced in mice injected once with an emulsion composed of 1-ml FCA and 1-ml phosphate-buffered saline (PBS) solution of 60-mg heat-killed *Mycobacterium butyricum* (Ram *et al.*, 1968). Female ICR mice weighing 20 to 25 g, aged 6 to 8 weeks, were injected intraperitoneally with 0.2 ml of the emulsion that was donated by Dr. Ishihara and Dr. Kawano of Yamaguchi University, Japan. The control mice were injected with PBS without the emulsion. Mice were sacrificed 3, 7, and 14 days after injection.

4. Detection of Components of Amyloid Fibrils and Macrophages

Antibodies of F4/80 and ER-TR9 were used as a marker of red pulp macrophages and marginal zone macrophages, respectively (Dijkstra *et al.*, 1985). The spleens of the mice were processed for immunohistochemical examinations as described in the Methods of our previous study (Kuroiwa *et al.*, 2003).

4.1. Detection of F4/80 Macrophages with Light Microscopy

The deparaffinized sections were incubated with rat antimouse F4/80 antibody (1 : 10 dilution, Serotec Co., UK) and reacted by the avidin–biotin complex methods (DAKO Co., Carpinteria CA, USA). The sections were colored with 3, 3'-diaminobenzidine-4HCl (DAB). The immunostained sections were observed with an Olympus AH 3 light microscope (Olympus, Tokyo, Japan).

4.2. Detection with Fluorescence Microscopy

The double immunofluorescence method was applied to the frozen sections of the spleen of the mouse (Kuroiwa *et al.*, 2003). Rat antimouse F4/80 antibody (1 : 50 dilution), rat antimouse ER-TR9 antibody (Biomedicals AG, Switzerland), anti-CSPG mouse monoclonal antibody (1 : 100 dilution, Sigma, St. Louis, MO, USA), and rabbit antimouse serum APC antibody (1 : 500 dilution, Calbiochem-Novabiochem Co., UK) were used as primary antibodies. Alexa Flour 594 labeled goat antirat IgG and IgM (1 : 400 dilution, Molecular Probes, Eugene, OR, USA), Alexa Flour 594 labeled goat antirabbit IgG (1 : 400 dilution, Molecular Probes), FITC conjugated goat antimouse IgG (1 : 100 dilution, Sigma), and Alexa Flour 488 labeled goat antirabbit IgG (1 : 400 dilution, Molecular Probes) were used as secondary antibodies. The pairs of primary and secondary antibodies are listed in Table 11.2-1. Sections were observed with an Olympus AX-70 fluorescence microscope (Olympus, Tokyo, Japan).

4.3. Detection with Confocal Laser-Scanning Microscopy

The double immunofluorescence method was applied to paraffin sections 7μm in thickness. The sections were incubated with a mixture of rat antimouse F4/80 antibody (1 : 50 dilution) and

Table 11.2-1. Pair of primary and secondary antibodies

Primary antibody	Secondary antibody
F4/80	Alexa 594
ER-TR9	Alexa 594
APC	Alexa 594* or Alexa 488
CSPG	FITC

F4/80, rat antimouse F4/80 antibody; ER-TR9, rat antimouse ER-TR9 antibody; APC, rabbit antimouse serum amyloid P component antibody; CSPG, antichondroitin sulfate proteoglycan mouse monoclonal antibody; Alexa 594, Alexa Flour 594-labeled goat antirat IgG and IgM; Alexa 594*, Alexa Flour 594-labeled goat antirabbit IgG; Alexa 488, Alexa Flour 488-labeled goat antirabbit IgG; FITC, FITC-conjugated goat antimouse IgG.

rabbit antimouse APC antibody (1:500 dilution) overnight at 4°. After washing several times, the sections were incubated with a mixture of Alexa Flour 594 labeled goat antirat IgG and IgM (1:400 dilution) and Alexa Flour 488 labeled goat antirabbit IgG (1:100 dilution). The immunostained sections were observed under a Leica TCS-SP2 confocal laser-scanning microscope (Leica, Tokyo, Japan).

4.4. Detection with Electron Microscopy

Pieces of spleen tissue were fixed with a solution of 1% each of paraformaldehyde and glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed with 1% osmium tetroxide in the phosphate buffer, and embedded in Epon. Ultrathin sections were made, stained with uranyl acetate followed by lead citrate, and observed with an Hitachi H-7500 electron microscope (Hitachi, Tokyo, Japan).

5. Induction of Amyloid Deposition in the Marginal Zone

Serum amyloid A levels were measured on days 3, 7, and 14 after injection of the emulsion. The level of SAA on day 3 was the highest, although amyloid deposition stained with Congo red was not observed with light microscopy. Serum amyloid A levels had gradually decreased by days 7 and 14 but were still higher than the normal level. The amyloid deposition detected by Congo red staining (red-green birefringence under polarized light) began to be localized in the marginal zone of white pulp in the spleen on day 7. The amyloid deposition area in the spleen became wider on day 14. F4/80 positive macrophages were scatter-distributed in the red pulp on day 7 (**Figure 11.2-1a; see color insert**) and accumulated in the amyloid deposition area on day 14 (**Figure 11.2-1b; see color insert**).

6. Time-kinetic Detection of Amyloid Components and Macrophages by Double Immunofluorescence Method

The localizations of F4/80 positive macrophages (red pulp macrophages), ER-TR9 positive macrophages (marginal zone macrophages), CSPG, and APC were observed with fluorescence microscopy on days 3, 7, and 14 after injection of the emulsion (**Figures 11.2-2–11.2-6; see color insert**). CSPG positive cells were found in the red pulp on day 3 (**Figure 11.2-2a; see color insert**). CSPG was localized in the cytoplasm of the cells that were F4/80 negative. CSPG was not yet distributed in the extracellular spaces of the marginal zone. On day 14, the majority of F4/80-positive macrophages in the marginal zone showed CSPG in their cytoplasm (**Figure 11.2-2b; see color insert**). CSPG was also detected in the amyloid deposition area in the marginal zone. Double staining of CSPG and APC indicated that some of the CSPG positive cells showed APC positive on day 3 (**Figure 11.2-3a; see color insert**). APC was not localized in the extracellular spaces of the marginal zone. On day 14, CSPG and APC were localized in the amyloid deposition areas (**Figure 11.2-3b; see color insert**). Therefore, we next examined the localizations of CSPG, APC, and ER-TR9-positive macrophages after emulsion injection (**Figures 11.2-4 and 11.2-5; see color insert**). On day 3, CSPG was not detected in the ER-TR9-positive macrophages (**Figure 11.2-4a; see color insert**), but all ER-TR9-positive macrophages examined were APC positive (**Figure 11.2-5a; see color insert**). On day 7, CSPG and APC were localized in the amyloid deposition area of the marginal zone. Furthermore, ER-TR9-positive macrophages, which were already APC positive on day 3, showed both CSPG

and APC positive (**Figures 11.2-4b and 11.2-5b; see color insert**). Amyloid P component was not localized in the F4/80 positive macrophages on day 3 (**Figure 11.2-6; see color insert**).

Immunolocalizations of APC and F4/80 positive macrophages were also observed on day 14 after the injection of the emulsion (**Figure 11.2-7; see color insert**). Serial sections at intervals of approximately 1.5 μ m observed with confocal laser-scanning microscopy revealed that APC in the amyloid deposition and F4/80 positive macrophages near the marginal zone localized separately (**Figure 11.2-7a and 11.2-7b; see color insert**). However, the macrophages near the amyloid deposition were sometimes seen in close connection with the APC (**Figure 11.2-7c and 11.2-7d; see color insert**).

7. Formation of Amyloid Fibrils

Amyloid fibrils are composed of a core covered by a layer of HSPG where AA protein filaments are found. The core is a microfibril-like structure where APC is enclosed in a tight helical structure by CSPG (Inoue and Kisilevsky, 1996; Inoue *et al.*, 1998b). AA amyloidosis occurs in patients who show high SAA levels, as part of the acute-phase response in a wide range of diseases (McAdam *et al.*, 1978; Sipe, 1978; Gillmore *et al.*, 2001). The defective clearance of SAA, a precursor of AA protein (McAdam *et al.*, 1978; Sipe, 1978; Inoue and Kisilevsky, 1996; Inoue *et al.*, 1998b, 2002), from the tissues by the decrease of proteolytic enzyme activities has been suggested as a trigger for amyloidogenesis in the extracellular matrix (Du *et al.*, 1990). In this study, SAA levels increased prior to amyloid deposition and gradually reduced during the course of amyloid formation. APC, CSPG, and HSPG, as well as SAA, are components of amyloid fibril, and are known to be acute phase reactants (McAdam *et al.*, 1978; Sipe, 1978; Snow *et al.*, 1991; Lyon *et al.*, 1993; Inoue and Kisilevsky, 1996; Inoue *et al.*, 1998b, 2002; Togashi *et al.*, 1997; Gillmore *et al.*, 2001). Acute and chronic inflammatory reactions play important roles in AA amyloidosis.

Previous studies have indicated that the formation of amyloid fibrils occurs in macrophages (Shirahama and Cohen, 1975; Uchino *et al.*, 1985; Takahashi *et al.*, 1989). The differences between ER-TR9-positive marginal zone macrophages and F4/80-positive red pulp macrophages were identified in this study. ER-TR9-positive macrophages are located exclusively in the marginal zone of white pulp and not in any other compartment (Dijkstra *et al.*, 1985). ER-TR9-positive macrophages showed APC positive on day 3 and changed to APC/CSPG double positive on day 7. Because APC and CSPG are the components of a core of amyloid fibrils, it has been suggested that ER-TR9-positive macrophages are associated with amyloid fibril formation.

Our immunohistochemical study shows that F4/80/ER-TR9-negative fibroblasts with APC and CSPG were also detected on day 3. AA protein and SAA have been observed in fibroblasts *in vitro*, in the splenic perifollicular reticuloendothelial cells and reticular cells *in vivo* after amyloid induction (Uchino *et al.*, 1985; Takahashi *et al.*, 1989; Chronopoulos *et al.*, 1994). Although fibroblasts and reticuloendothelial cells are not strictly distinguishable under electron microscopy, a large number of amyloid fibrils extended from fibroblasts into the extracellular spaces in the marginal zone on day 14 (**Figure 11.2-8; see color insert**). The amyloid fibrils extended diagonally into the extracellular spaces from fibroblasts (**Figure 11.2-8b; see color insert**). The amyloid fibrils extended and were well orientated along the cytoplasmic process of the fibroblasts (**Figure 11.2-8a; see color insert**) just as described in other reports (Takahashi *et al.*, 1989; Chronopoulos *et al.*, 1994). The fibroblasts contained a considerable number of lysosomes and a rough endoplasmic reticulum in the cytoplasm. This finding resembles collagen fibrogenesis in fibroblasts (Trelstad and Hayashi, 1979; Birk and Trelstad, 1984), and these results suggest that APC/CSPG-positive fibroblasts and/or ER-TR9 are related to the formation of the central core of amyloid fibrils. The amyloid fibrils are extended by these cells in the extracellular spaces of the marginal zone in the spleen.

8. Resorption of Amyloid Fibrils

One prominent finding in this study was the rapid accumulation of F4/80-positive red pulp macrophages in the amyloid deposition area. F4/80-positive macrophages were APC/CSPG double negative in controls, but became APC/CSPG positive after migration to the marginal zone near the deposition of amyloid fibrils. At the ultrastructural level, macrophages in the marginal zone showed many lysosomal dense bodies and phagosomes containing fibrous structures (Chronopoulos *et al.*, 1994). Similarly, F4/80-positive macrophages in the amyloid deposited area on day 14 showed lysosomal dense bodies, phagolysosomes, rough endoplasmic reticulums, and Golgi apparatuses (**Figure 11.2-9; see color insert**). The macrophages contained fibrous structures 150 to 350 nm in width within the membrane-bound, oval to fusiform dense bodies ultrastructurally comparable to the lysosomal derived structure. The fibrous structures in the dense bodies were 10 to 12 nm in width and had a feature identified as amyloid fibrils. The amyloid fibrils did not extend from the macrophages into the extracellular spaces. It is suggested from these results that F4/80-positive red pulp macrophages migrate to the marginal zone during amyloidogenesis and then participate in the resorption of the amyloid fibrils. The exact mechanisms of macrophage migration have not been clarified. Recently, increases of tumor necrosis factor- α (TNF- α) and macrophage colony-stimulating factor (M-CSF) levels in systemic amyloidosis (amyloid A) have been reported (Rysava *et al.*, 1999). These cytokines might stimulate the migration and the phagocytosis of macrophages. Further examination of murine amyloidosis over 14 days after injection is necessary to reveal the intimate process of amyloid fibrils by macrophages.

9. Abbreviations

AA	Amyloid A
AEF	amyloid-enhancing factor
APC	amyloid P component
CSPG	chondroitin sulfate proteoglycan
DAB	3,3'-diaminobenzidine-4HCl
HSPG	heparan sulfate proteoglycan
M-CSF	macrophage colony-stimulating factor
PBS	phosphate-buffered saline
SAA	serum amyloid A
TNF- α	tumor necrosis factor- α

References

- Benson, M.D., and Kleiner, E. (1980). Synthesis and secretion of serum amyloid protein A (SAA) by hepatocytes in mice treated with casein. *J. Immunol.* 124:495–499.
- Birk, D.E., and Trelstad, R.L. (1984). Extracellular compartments in matrix morphogenesis: collagen fibril, bundle, and lamellar formation by corneal fibroblasts. *J. Cell Biol.* 99:2024–2033.
- Chronopoulos, S., Laird, D.W., and Ali-Khan, Z. (1994). Immunolocalization of serum amyloid A and AA amyloid in lysosomes in murine monocytoic cells: confocal and immunogold electron microscopic studies. *J. Pathol.* 173:361–369.
- Dijkstra, C.D., Van Vliet, E., Dopp, E.A., Van Der Lelij, A.A., and Kraal, G. (1985). Marginal zone macrophages identified by a monoclonal antibody: characterization of immuno- and enzyme-histochemical properties and functional capacities. *Immunology* 55:23–30.

- Du, T., and Ali-Khan, Z. (1990). Pathogenesis of secondary amyloidosis in an alveolar hydrated cyst-mouse model: histopathology and immuno/enzyme-histochemical analysis of splenic marginal zone cells during amyloidogenesis. *J. Exp. Pathol.* 71:313–335.
- Gillmore, J.D., Lovat, L.B., Persey, M.R., Pepys, M.B., and Hawkins, P.N. (2001). Amyloid load and clinical outcome in AA amyloidosis in relation to circulating concentration of serum amyloid A protein. *Lancet* 358:24–29.
- Glenner, G.G., Terry, W.D., and Isersky, C. (1973). Amyloidosis: its nature and pathogenesis. *Semin. Hematol.* 10:65–86.
- Inoue, S., and Kisilevsky, R. (1996). A high resolution ultrastructural study of experimental murine AA amyloid. *Lab. Invest.* 74:670–683.
- Inoue, S., Kuroiwa, M., Ohashi, K., Hara, M., and Kisilevsky, R. (1997). Ultrastructural organization of hemodialysis-associated β 2-microglobulin amyloid fibrils. *Kidney Int.* 52:1543–1549.
- Inoue, S., Kuroiwa, M., Saraiva, M.J., Guimarães, A., and Kisilevsky, R. (1998a). Ultrastructure of familial amyloid polyneuropathy amyloid fibrils: examination with high-resolution electron microscopy. *J. Struct. Biol.* 124:1–12.
- Inoue, S., Kuroiwa, M., Tan, R., and Kisilevsky, R. (1998b). A high resolution ultrastructural comparison of isolated and in situ murine AA amyloid fibrils. *Amyloid Int. J. Exp. Clin. Invest.* 5:99–110.
- Inoue, S., Kuroiwa, M., and Kisilevsky, R. (1999). Basement membranes, microfibrils and β amyloid fibrillogenesis in Alzheimer's disease: high resolution ultrastructural findings. *Brain Res. Rev.* 29:218–231.
- Inoue, S., Kuroiwa, M., and Kisilevsky, R. (2002). AA protein in experimental murine AA amyloid fibrils: a high resolution ultrastructural and immunohistochemical study comparing aldehyde-fixed and cryofixed tissues. *Amyloid Int. J. Exp. Clin. Invest.* 9:115–125.
- Kuroiwa, M., Aoki, K., and Izumiya, N. (2003). Histological study of experimental murine AA Amyloidosis. *J. Elect. Microsc.* 52:407–413.
- Linder, E., Anders, P.F., and Natvig, J.B. (1976). Connective tissue origin of the amyloid-related protein SAA. *J. Exp. Med.* 144:1336–1346.
- Lyon, A.W., Anastassiades, T., and Kisilevsky, R. (1993). *In vivo* analysis of murine serum sulfate metabolism and splenic glycosaminoglycan biosynthesis during acute inflammation and amyloidosis. *J. Rheumatol.* 20:1108–1113.
- McAdam, K.P.W.J., Elin, R.J., Sipe, J.D., and Wolff, S.M. (1978). Changes in human serum amyloid A and C-reactive protein after etiochemolanolene-induced inflammation. *J. Clin. Invest.* 61:390–394.
- Ram, J.S., Delellis, R.A., and Glenner, G.G. (1968). Amyloid. III. A method for rapid induction of amyloidosis in mice. *Int. Arch. Allergy* 34:201–204.
- Rysava, R., Merta, M., Tesar, V., Jirsa, M., and Zima, T. (1999). Can serum amyloid A or macrophage colony stimulating factor serve as marker of amyloid formation process? *Biochem. Mol. Biol. Int.* 47:845–850.
- Shirahama, T., and Cohen, A.S. (1973). An analysis of the close relationship of lysosomes to early deposits of amyloid. Ultrastructural evidence in experimental mouse amyloidosis. *Am. J. Pathol.* 73:97–114.
- Shirahama, T., and Cohen, A. S. (1975). Intralysosomal formation of amyloid fibrils. *Am. J. Pathol.* 81:101–116.
- Sipe, J.D. (1978). Induction of the acute-phase serum protein SAA requires both RNA and protein synthesis. *Br. J. Exp. Pathol.* 59:305–310.
- Sipe, J.D. (1994). Amyloidosis. *Critical Rev. Clin. Lab Sci.* 31:325–354.
- Snow, A.D., Bramson, R., Mar, H., Wight, T.N., and Kisilevsky, R. (1991). A temporal and ultrastructural relationship between heparan sulfate proteoglycans and AA amyloid in experimental amyloidosis. *J. Histochem. Cytochem.* 39:1321–1330.
- Takahashi, M., Yokota, T., Yamashita, Y., Ishihara, T., and Uchino, F. (1985). Ultrastructural evidence for the synthesis of serum amyloid A protein by murine hepatocytes. *Lab. Invest.* 52:220–223.
- Takahashi, M., Yokota, T., Kawano, H., Gondo, T., Ishihara, T., and Uchino, F. (1989). Ultrastructural evidence for intracellular formation of amyloid fibrils in macrophages. *Virchows Arch. [A]* 415:411–419.
- Tatsuta, E., Sipe, J.D., Shirahama, T., Skinner, M., and Cohen, A.S. (1983). Different regulatory mechanisms for serum amyloid A and serum amyloid P synthesis by cultured mouse hepatocytes. *J. Biol. Chem.* 258:5414–5418.
- Togashi, S., Lim, S.-K., Kawano, H., Ito, S., Ishihara, T., Okada, Y., Nakano, S., Kinoshita, T., Horie, K., Episkopou, V., Gottesman, M.E., Costantini, F., Shimada, K., and Maeda, S. (1997). Serum amyloid P component enhances induction of murine amyloidosis. *Lab. Invest.* 77:525–531.
- Trelstad, R.L., and Hayashi, K. (1979). Tendon collagen fibrillogenesis: intracellular subassemblies and cell surface changes associated with fibril growth. *Dev. Biol.* 71:228–242.
- Uchino, F., Takahashi, M., Yokota, T., and Ishihara, T. (1985). Experimental amyloidosis: role of the hepatocytes and Kupffer cells in amyloid formation. *Appl. Pathol.* 3:78–87.

Visualization of Protein Deposits *In Vitro*

Reporters of Amyloid Structure

Harry LeVine, III

1. Abstract

Reporters of amyloid fibril structure have contributed greatly to our understanding of the biochemical processes of fibril formation. Congo Red and Thioflavine T have been used to discover agents that disrupt fibril formation. Now labeled tight binding ligands are being assessed for their utility as imaging agents to diagnose amyloid deposition and to monitor the efficacy of therapeutic regimens. Antibodies selective for the fibrils or amyloidogenic forms of the proteins originally defined by microscopy and by small molecule probes are presently being used to investigate the involvement of these species in the pathology of disease. Some of these immunological tools may also have therapeutic utility in depleting toxic forms of the proteins. Further development of these probes will improve diagnosis and expedite the development of therapeutic strategies for the increasing number of recognized diseases of protein misfolding.

2. Common Elements of Amyloid Fibrils

Atomic level resolution structure of amyloid fibrils has remained an elusive goal since Virchow's original description in 1854 of the waxy deposits in tissue that stained in a characteristic way with certain dyes (Sipe and Cohen, 2000). Congo Red staining with a metachromatic shift in absorbance and birefringence implied regular structural organization in the deposits from all amyloid fibrillar forms regardless of primary amino acid sequence (Kelenyi, 1967; Turnell and Finch, 1992; Carter and Chou, 1998). These peculiar dye binding properties and refraction of polarized light became defining features for amyloid fibrils. Additional evidence for repeating structure came from X-ray diffraction of oriented fibrils. Noncrystalline, yet regular enough to produce interpretable diffraction patterns, a common overall structural organization was evident, that could potentially account for the similar dye binding properties (Sunde *et al.*, 1997 Serpell *et al.*, 2000).

Synthetic amyloid fibrils share enrichment in β -sheet structure detectable by circular dichroism and by Fourier transform infrared (FTIR) spectroscopy. FTIR (Choo *et al.*, 1996) and linear dichroism measurements on plaques and cerebrovascular amyloid (Jin *et al.*, 2003) *in situ* verify this in tissue sections. Solid-state and solution-phase NMR studies have recently provided an atomic level perspective on the overall organization of Amyloid β peptide (A β) fibrils. The A β peptides can evidently associate in an antiparallel or parallel fashion depending on the length of the sequence (Tycko and Ishii, 2003). A hallmark of all amyloid fibrils is their resistance to degradation by a variety of proteases with general or sequence specificities (Nordstedt *et al.*, 1994). This resistance has implications for the *in vivo* persistence of amyloid plaques assembled from fibrils. The N-terminus of fibrillar A β is susceptible to proteolysis, while much of the rest is less exposed

or wholly buried (Kheterpal *et al.*, 2001). The tightness of the structure is corroborated by H/D exchange studies of the amide hydrogens with solvent. A proposed precursor to fibrils, A β protofibrils are partially exposed while mature fibrils are more fully protected (Kheterpal *et al.*, 2000, 2003a, 2003b). A similar situation is found with β_2 -microglobulin in which a narrow core of the peptide is shielded from solvent in immature fibrils. This protected region is greatly expanded in mature fibrils (Yamaguchi *et al.*, 2004). Although these measures do not provide an atomic level picture of the structure of the assembled peptide, the residues that are accessible to solvent can be identified.

The *in vitro* formation of amyloid fibrils from purified or synthetic monomeric protein or peptide also appears to follow a common mechanism. For a number of amyloid proteins, the different stages have been visualized by electron microscopy (EM) and atomic force microscopy (AFM) (Ionescu-Zanetti *et al.*, 1999; Kad *et al.*, 1999, 2003; Conway *et al.*, 2000; Khurana *et al.*, 2003; Legleiter and Kowalewski, 2003; Wang *et al.*, 2003). Although individual proteins differ somewhat in the fine details, amyloid fibril formation proceeds through nucleated polymerization with either on- or off-pathway oligomeric intermediates to protofibrils and finally fibrils (Harper *et al.*, 1997; Goldsbury *et al.*, 1999; Ionescu-Zanetti *et al.*, 1999; Modler *et al.*, 2003; Gibson *et al.*, 2004; Green *et al.*, 2004). This assembly is accompanied by decreased solubility and increased resistance to proteolysis.

3. Probes in Which Amyloid Fibrils Induce Changes

Distinguishing amyloid fibril structure from that of normally folded nonfibrillar cognate proteins that may contain β -sheets is important for pathological characterization and for *in vitro* biochemical studies of fibril assembly. It is also likely to be of major utility in detection of disease lesions and in monitoring of the efficacy of potential disease-modifying therapeutic interventions. The following sections provide an overview of a variety of reporters of amyloid structure, most of which have been developed for use with the Alzheimer's A β peptide, but many of which are or could be useful for studying amyloid fibrils comprised of other entities.

3.1. Probes of Fibril Formation

The azobenzene cotton dye Congo Red (C.I. 22120) is the classic histologic agent for the detection of amyloid fibrils in tissue sections, and is also widely used *in vitro*. Binding to the regular structure of an amyloid fibril induces birefringence in light absorption as well as a metachromatic shift in the absorbance, producing a characteristic apple green "Maltese cross" of amyloid plaques under polarized illumination. The number of Congo Red molecules bound can be determined by difference absorption spectroscopy in solutions containing fibrils and dye (Klunk *et al.*, 1999). Congo Red affinity for A β fibrils and plaques is only moderate. High affinity, brain penetrant, fluorescent, and more metabolically stable styrene analogs of Congo Red such as X-34 (Styren *et al.*, 2000) and K114 (Crystal *et al.*, 2003) react with a variety of different amyloid fibrils and are being considered as *in vivo* imaging agents for Alzheimer's disease (AD) plaques.

The benzothiazole dyes Thioflavine S (ThS) (C.I. 49010) and Thioflavin T (ThT) (C.I. 49005) have been used since the 1960s for the histological demonstration of amyloid fibrils. The free dyes share the benzothiazole fluorophore (λ_{ex} ~335 nm; λ_{em} ~490 nm) but differ significantly from other benzothiazoles when they are bound to amyloid fibrils (Naiki *et al.*, 1989; LeVine, 1993, 1999). Thioflavine S is a complex mixture of methylated and sulfonated primuline (C.I. 49000) polymers.

Its emission intensity is enhanced in the presence of amyloid fibrils (Naiki *et al.*, 1989). Despite being a heterogeneous mixture, the anionic ThS is commonly used for histology. Its cationic counterpart, ThT, on the other hand, is a single-molecular species of low molecular weight. In contrast to ThS, binding of ThT to amyloid fibrils induces a red shift of over 100 nm in the excitation spectrum of the bound dye (346 nm $\rightarrow$ 450 nm) with a minimal shift in the emission maximum (490 nm). Because ThT is a single species and the shift in excitation away from the free dye light absorption reduces background fluorescence, ThT is most frequently chosen for biochemical studies of the formation of amyloid fibrils. The molecular basis for the apparent lowering of the ground state (excitation) phenomenon is not understood. A similar effect on the excitation spectrum of ThT can be produced in the absence of amyloid fibrils by high concentrations of polyalcohols (glycerol, ethylene glycol) that cannot be attributed to viscosity effects or dielectric constant (LeVine, 1995). Multimerization of the dye is unlikely to explain the shift, as the fluorescence characteristics of ThT are not dependent on free dye concentration.

The interaction of ThT with individual amyloid protein fibrils shows some effect of the primary sequence as the apparent dye affinity varies from 10^{-8} to 10^{-5} M and the pH dependence of ThT interactions range from pH 4–9 (LeVine, 1995). Not all amyloid fibrils binding ThT display the same maximal intensity. Amylin (IAPP) (20–29), which is fibrillar by EM and Congo Red positive, gives only a faint signal with ThT. ThT association with A β fibrils assessed by the fluorescence change compared to direct binding is markedly substoichiometric, with the particulars depending on the A β sequence in the fibrils. The quantum yields of the binding sites seem to differ, and there may be a substantial fraction of ThT binding sites on fibrils that does not produce amyloid-dependent fluorescence.

DDNP is one of a family of naphthyl malononitriles that bind tightly to A β fibrils and neurofibrillary tangles. The emission intensity of DDNP is increased upon binding, with minimal change in either the excitation or emission wavelengths. Aqueous solutions of these molecules are weakly fluorescent due to efficient electronic relaxation by a molecular rotor mechanism involving the malononitrile. The emission intensity is enhanced by the immobilization of the malononitrile moiety, an effect that can be mimicked with the free dye by raising the viscosity of the medium. The dye is postulated to bind in an aqueous nanotube core of the amyloid fibrils (Perutz *et al.*, 2002). The high affinity of these interactions has been exploited to label amyloid fibrils and neurofibrillary tangles *in vivo*, and will be covered in the next section on tight binding affinity agents for amyloid fibrils.

3.2. Probes of Soluble A β Monomer Conformation

Most of the probes for A β conformation are for the fibrillar peptide. This is not surprising, as the supramolecular organization of the fibrils provides ample surface area and a stable structure for binding sites. The soluble forms of the A β peptide, including oligomeric forms, are much more dynamic. No small molecule ligands have been identified that report strictly on oligomeric species. Such a tool would be very helpful in detecting and characterizing these species, which seem to possess toxicity for cells and could be the missing link between the visible pathology of plaques and tangles in AD and neurodegeneration.

The A β species with the most dynamic structure is the monomeric peptide. Unexpectedly, the hydrophobic dye bis-ANS undergoes an enhancement in fluorescence *in vitro* when bound to monomeric A β but not when bound to fibrils (LeVine, 2002). The largest enhancement is observed when the A β peptide is in an α -helical conformation and the least when in a β -sheet conformation. The dye may be detecting the α -helical intermediate in fibril formation suggested by some studies (Kirkitadze *et al.*, 2001). Interconversion of A β 40 between the random coil, α -helix, and β -sheet can

be followed by bis-ANS fluorescence in purified systems. The central core of the peptides A β (10–35) and A β (12–28) account for the bulk of the reactivity. Hydrophobic segments of the A β peptide do not induce these changes in bis-ANS (LeVine, 2002).

4. Tight Binding Probes for Amyloid Imaging

Amyloid proteins accumulate in anatomical locations characteristic of the protein component and the disease. These deposits occur as either amorphous assemblages of amyloidogenic and associated proteins or as morphologically distinctive fibrils with characteristic periodicity and defined substructure depending on the amyloid protein constituent. Extracellular matrix elements are believed to participate in forming the original nidus (Kisilevsky *et al.*, 1992; Kisilevsky and Fraser, 1996). In peripheral amyloid accumulations they constitute a significant proportion of the deposits, along with various proteoglycans, gangliosides, and a series of proteins such as Serum Amyloid P protein (SAP) and apolipoprotein E (ApoE) (Brandan and Inestrosa, 1993; McGeer *et al.*, 1994). These accessory components are thought to influence the structure and stability of amyloid deposits, altering their properties from those of amyloid fibrils formed *in vitro* or deposited in their absence.

Amyloid deposits are generally associated with a disease state by direct physical interference with organ function. They may also elicit pathology through secondary immune responses or other toxic mechanisms (Christen, 2000; Miyata *et al.*, 2000; McGeer and McGeer, 2002, 2003; Alessenko *et al.*, 2004; Huang *et al.*, 2004). Diagnosis before irreversible damage occurs is problematic, because the deposits are focal and hard to sample (Olsen *et al.*, 1999; Ando *et al.*, 2003; Ansari-Lari and Ali, 2004). Hence, there is an outstanding need to noninvasively quantify amyloid load *in situ*. Magnetic resonance imaging (MRI) can detect large deposits, frequently from their displacement of tissue arrangement. Small deposits, however, such as the A β plaques in AD brain or the A β angiopathy along cerebral blood vessels require enhancement. A variety of molecules with selectivity and affinity for amyloid plaques are being investigated for potential use as imaging agents in diagnosing disease, monitoring disease progression, and for assessing efficacy of therapeutic interventions.

4.1. Distinction Between Plaques and Neurofibrillary Tangles

Two different misfolded protein pathologies are prominent in AD: the A β peptide in plaques, and tau protein in neurofibrillary tangles (NFTs). To this may be added α -synuclein in the form of Lewy Bodies, which can be comorbid with plaques and tangles in AD but also occur alone associated with a distinct dementia in Diffuse Lewy Body disease. Plaques and tangles are both involved in AD but follow different time courses and patterns of occurrence, so it may be important to be able to distinguish them. Because both deposits share elements of amyloid fibril structure, their crossreactivity with imaging agents is a potential area of concern. Quantifying (plaques + tangles) in different brain areas may be less sensitive to disease stage or therapeutic intervention than determining the pathologies separately.

Tagging approaches for imaging fall into four categories: binding proteins, small molecule ligands, cognate peptide/protein, and antibodies.

4.2. Binding Proteins

Proteins that show an extraordinary predilection for binding to amyloid fibrils are natural candidates for imaging agents. Low background binding to other sites and favorable wash in/wash out kinetics are also required for imaging contrast as well as low toxicity.

Serum amyloid P protein is associated with amyloid deposits comprised of many different proteins. This member of the pentraxin family has been shown to be useful to detect deposits and to monitor their turnover in cases of peripheral amyloidosis (Pepys *et al.*, 2002). Serum amyloid P protein is not found in senile plaques in AD brain, and radioactive SAP does not label plaques in AD brain when administered peripherally, which may be because SAP does not cross the blood–brain barrier.

Basic fibroblast growth factor (bFGF) localizes to senile plaques and tangles in AD brain (Cummings *et al.*, 1993; Friedland *et al.*, 2000). ^{125}I -labeled bFGF is taken up by the plaques in hA β PP transgenic mice when applied intranasally, and accumulates in the transgenic animals but not in nontransgenic animals and localizes to amyloid plaques (Blass, 2002; Shi *et al.*, 2002). The molecular basis for the selective interaction with plaques is unknown, and labeling remains to be demonstrated in humans.

Apolipoprotein E has an etiologic connection to AD. The $\beta 4$ isoform is significantly overrepresented in AD patients (Poirier *et al.*, 1993, 1995), and appears linked to amyloid load (Walker *et al.*, 2000). *In vitro*, apoE interacts substoichiometrically at nM affinity with A β fibrils (LeVine, 2000). Although apoE is transported into the brain readily, it binds to and is internalized by a variety of cell types through the lipoprotein receptor-like receptor family (Kang *et al.*, 2000; Ladu *et al.*, 2000; LaDu *et al.*, 2000; Poirier, 2000), which rules it out as a plaque imaging agent.

4.3. Small Molecule Binders

The diazo dye Congo Red is a 697-Da, nonbrain penetrant, rapidly metabolized probe for amyloid structure at micromolar concentrations. The affinity of the diazostilbene nucleus for amyloid fibrils and brain penetrance were improved by reducing the charge on the molecule as with Chysamine G (Klunk *et al.*, 1994). A series of styryl substitutions for the diazo linkages and further charge modification (X-34; BSB, K114) improved the pharmacokinetics and the affinity for amyloid fibrils and produced fluorescent compounds that localize to plaques when injected into hA β PP transgenic mice (Skovronsky *et al.*, 2000). Other amyloid-binding pharmacophores have been labeled, and their suitability for brain imaging evaluated (Kung *et al.*, 2003). More optimization is required.

Another potential imaging agent, BF-108, is based on the acridine dyes (Suemoto *et al.*, 2004). *In vitro*, and *in vivo* exposure of hA β PP transgenic mice indicate that the compound penetrates the blood–brain barrier and stains plaques and blood vessel amyloid. In AD brain sections plaques, amyloidotic cerebrovasculature, and neurofibrillary tangles are all labeled.

DDNP, despite a hydrophobicity and structure that imply nonspecific staining properties, remarkably selectively stains A β amyloid fibril structure at low concentrations. Some positron emission tomography (PET) imaging studies in animals and in human patients with ^{18}F -labeled FDDNP have been promising (Shoghi-Jadid *et al.*, 2002).

The property of staining both amyloid fibrils and tau filaments in NFTs is also found among certain ligands that incorporate the benzothiazole nucleus. The permanently charged ThT and ThS change their fluorescence characteristics upon binding to amyloid plaques, A β fibrils, tau fibrils, and NFTs. Notably, the fluorescence of their tighter binding derivatives is unaffected, suggesting a different binding site. These smaller, uncharged, or weakly charged molecules bind several orders of magnitude more tightly than ThT or ThS and are brain-penetrant. Multiphoton optical microscopy through a window in the skull showed localization of these compounds to amyloid plaques in living transgenic hA β PP animals (Bacskaï *et al.*, 2003). The most extensive published studies have been with ^{14}C -labeled BTA-1 (PIB1), which reproducibly quantifies plaque load and shows an increase in signal with disease progression in the small number of patients that have been examined. Even with the limited resolution of clinical PET imagers, plaque-laden areas can readily be distinguished and

a volumetric measure of plaque load obtained (Klunk *et al.*, 2004). ^{18}F -labeled benzothiazole IMPY derivatives have also shown useable kinetics in animal studies (Cai *et al.*, 2004). Selectivity for senile plaques over NFTs can be achieved with some of these small molecules. A benzothiazole derivative TDZM binds selectively to plaques but not tangles (Lee, 2002; Kung *et al.*, 2003). This selectivity could be an issue when used to monitor clinical efficacy of amyloid reduction strategies as noted in a previous section.

Small-molecule single photon emission computed tomography (SPECT) or PET radioligands binding to amyloid plaques and tangles seem to be the most advanced imaging probes, due in part to their advantages in brain penetration and clearance. The plaque/tangle specificity issue remains to be fully addressed.

5. The Phenomenon of Cognate Peptide Recognition

Amyloid fibril formation and fibril growth are protein folding events requiring structural recognition. Although the overall organization of amyloid fibrils seems to be similar when viewed by various microscopies, spectroscopies, diffraction, and by certain conformation-sensitive antibodies, there remains an exquisite selectivity for binding of the monomer, and even particular conformations of the monomer (O'Nuallain *et al.*, 2004) to preexisting fibrils. The basis for this is incompletely understood at the molecular level. A series of studies by Maggio and colleagues (Tseng *et al.*, 1999; Esler *et al.*, 2000a, 2000b) and others (Cannon *et al.*, 2004; O'Nuallain *et al.*, 2004) have elucidated some mechanistic details for the A β peptide including a multistep process for binding and conformational conversion.

There is some question as to whether all of the details of fibril formation derived from studies of soluble synthetic peptide are physiologically relevant. For practical reasons, fibril formation is carried out in pure peptide systems at concentrations vastly in excess of the observed *in vivo* concentrations of A β . Fibril extension can be carried out in the presence of exogenous proteins and even in crude homogenates of AD brain or on AD brain slices at physiological concentrations of A β and below (Maggio *et al.*, 1992; Esler *et al.*, 1999). Congo Red does not inhibit this reaction, while it potently blocks fibril formation *in vitro* (Esler *et al.*, 1997). The question is more likely, how could fibril extension *not* be an *in vivo* reaction, although its relative contribution might depend on other factors. A β fibril extension type processes have received less attention for other amyloidogenic proteins in disease tissue, although distinct binding and conversion events have been suggested for prions (Chien and Weissman, 2001; Baskakov *et al.*, 2002; Chien *et al.*, 2003; Scheibel *et al.*, 2004).

Amyloid fibrils segregate to form relatively homogeneous polymers even in mixtures. Truncations and modifications seem to be mostly postassembly events, although this has not been widely studied. Such a scenario involving templating now accepted for prions may also play out to various extents for amyloid fibrils as well. The selectivity of prions is evidenced by the existence of "strains" displaying different biological phenotypes formed from chemically identical polypeptide chains. Antibody and resistance to denaturants indicate that the strain differences are conformation-dependent (Peretz *et al.*, 2001, 2002; Leclerc *et al.*, 2003).

The apparent selectivity of cognate amyloidogenic peptides and proteins for their fibrils has been utilized to label A β deposits *in vitro* and *in vivo*. Modification of the A β peptide with polyethylene glycol (Kurihara and Pardridge, 2000; Lee *et al.*, 2002) and diamines such as putrescine (Poduslo *et al.*, 2002) increases uptake into the brain and binding to plaques *in vitro* and in transgenic hA β PP mice. Soluble oligomeric forms of A β in CSF are labeled by A β peptides (Pitschke *et al.*,

1998). Prion oligomers are similarly labeled by cognate prion proteins, and do not crosslabel with A β peptide and vice versa (Bieschke *et al.*, 2000).

These modified peptides labeled with SPECT radioisotopes or gadolinium-chelate complexes for MRI are being considered to detect plaques in AD patients. One advantage of the peptides as probes is that they only label the amyloid plaques, not the NFT pathology.

6. Conformation-Dependent Antibodies

The exquisite specificity of antibodies for the primary amino acid sequence of a peptide or protein antigen is a widely recognized property that is used to advantage to recognize that sequence in a mixture of other peptide sequences. Immunostaining localizes that sequence in tissue sections to pinpoint local concentrations of that material. Quantitative immunoassays can determine the total amount of that sequence in a sample.

Many antibody epitopes consist of a linear sequence of five to eight amino acids. The standard procedure for raising antibodies with a short peptide conjugated to a carrier protein most often yields linear epitopes that are fine for many purposes such as detecting denatured proteins on Western blots. Another class of recognition element is the conformational epitope. These antibodies recognize a folded peptide sequence that may consist of discontinuous segments of the primary amino acid sequence that are brought together in space by the folding of that sequence. These antibodies are rarer, and are produced by immunizing with large segments or the full-length protein. Conformational epitopes are typically not blocked by linear peptide sequences.

To draw meaningful conclusions about the role of various conformational forms of A β with an immunological probe the specificity of an antibody must be carefully determined. This is more difficult for A β and molecules with similar properties than for most antigens. Preparations of monomers, oligomers, protofibrils, and fibrils are mixtures of the different forms. The equilibrium between these species can result in endogenous crosscontamination regardless of the rigor of the original isolation. Adsorption of the different forms to one another is another concern. The potential for cross-reactivity with other species of the antigen must be accounted for in interpreting results. This is particularly important in immunohistology, because the amount of antigen at a location is rarely determined, only its presence. It is important to realize that even affinity-purified polyclonal antibodies are a mixture. They are antibodies derived from a population of B-cell clones representing a range of specificities. A minor component or a relatively weak affinity antibody could react with a tissue or cellular structure giving the impression of a positive signal. Monoclonal antibodies, by definition, should react only with a single epitope, although monoclonal antibody affinity is frequently lower than for polyclonal antibodies.

6.1. Clinical Utility

Immunization with A β peptide sequences in transgenic mice overexpressing hA β PP surprisingly prevented formation of the β -amyloid plaques that normally develop in the brains of these animals upon aging (Schenk *et al.*, 1999; Games *et al.*, 2000; Frenkel *et al.*, 2003). Passive immunization with anti-A β antibodies was also effective in the transgenic hA β PP mice (Bard *et al.*, 2000; Chauhan and Siegel, 2003). Subsequent clinical trials in humans lowered A β levels, and based on the analysis of one AD subject who died, may have reduced plaque load and improved cognitive status (Hock *et al.*, 2003; Nicoll *et al.*, 2003). However, an unanticipated high incidence

of immune encephalitis forced termination of the trial (Orgogozo *et al.*, 2003; Ferrer *et al.*, 2004). Further study suggested that the isotype of the antibody produced (Bard *et al.*, 2003) and the form of A β targeted by the antibody generated by the immunization may govern the triggering of the adverse immunological reaction (Sigurdsson *et al.*, 2001). Thus, it is of more than academic interest to identify the proper form of A β peptide against which to raise antibodies and the type of immune response elicited.

6.2. Conformational Epitopes

An example of an antibody that distinguishes between conformations of monomeric A β is 6F/3D, a monoclonal antibody raised against the sequence 9–14. Peptide preexposed to pHs between 3 and 6 under conditions where there is no aggregation and then immobilized to freeze the conformation, does not react with 6F/3D in an ELISA format, although other epitopes such as the N-terminus are unaffected (Matsunaga *et al.*, 2002; LeVine, 2003). The epitope can be regenerated by treating the soluble peptide at alkaline pH > 8. This reactivity may simply reflect epitope exposure, as suggested by the fluorescence characteristics of a tryptophan-substituted mutant Y10W (LeVine, 2003). An interesting antibody would recognize the epitope generated at acidic pH, which could detect A β peptide that had been exposed to acidic conditions in the endosomal or lysosomal compartments.

6.3. Antifibrillar Antibodies

To probe the supramolecular structure of assembled species of A β , antibodies have been raised against A β fibrils. WO-1 and WO-2 are IgM subclass antibodies that react with fibrils of A β , TTR, IAPP, β 2-microglobulin, and polyglutamine (O’Nuallain and Wetzel, 2002). The IgM polymeric structure and tendency to aggregate make them a challenge to use for histology. The basis for their selectivity for fibrils is unexplained, although their multimeric form is unlikely to be involved because other anti-A β IgM antibodies do not show enhanced fibril reactivity. The reactivity with amyloid fibrils comprised of proteins of nonhomologous sequences parallels the similarities in fibril structure seen with the electron and atomic force microscopes. Another antibody raised against A β fibrils, R286, an IgG, demonstrates at least a 1000-fold selectivity for fibrillar A β over monomeric material (Miller *et al.*, 2003). Unlike the other antifibrillar antibodies, this antibody recognizes an epitope in the 1–16 sequence of A β in the fibrils. Perhaps the antibody is recognizing something about the way multiple epitopes in 1–16 are presented in fibrils, or maybe a particular conformation adopted by a more constrained 1–16 sequence in the fibril context.

The assembled fibrils from other proteins implicated in chronic neurodegenerative disease similarly elicit fibril-specific antibody responses (Korth *et al.*, 1999; Matsunaga *et al.*, 2001). Pathologic conformation-specific monoclonal antibodies to tau such as Alz50 and MC-1 (Ksiezak-Reding *et al.*, 1995; Jicha *et al.*, 1997a, 1997b, 1999) recognize folded forms of the tau protein that are associated with aberrant phosphorylation and NFT pathology. Phosphorylation-specific antibodies are generally not conformational antibodies in that they recognize the linear phosphorylated sequence, although for the proline-directed protein kinases, phosphoserine and phosphothreonine stabilize a *cis*-peptide bond conformation adjacent to the proline (Schutkowski *et al.*, 1998).

For the polyglutamine diseases such as Huntington’s disease, the antibody 1C2 recognizes the pathological conformation of the polyglutamine stretch (Perez *et al.*, 1999), while other antibodies recognize extended polyglutamine stretches (Trottier *et al.*, 1995). 1C2 has been valuable in identifying insoluble polyglutamine inclusions in neuronal nuclei in Huntington’s, and the various cerebellar ataxias. Very little is known about the structure of the deposited material. Fibrillar structures are less

obvious in the inclusions than in AD amyloid plaques, but they are ThT/ThS positive (Chen *et al.*, 2002). Water-filled nanotubes have been suggested as models for polyglutamine “polar zippers” (Perutz *et al.*, 2002).

6.4. Antioligomer Antibodies

Multimeric species of A β not yet assembled into fibrils have a distinctive structure detectable by physical techniques and by EM and AFM (Stine *et al.*, 1996; Goldsbury *et al.*, 1999; Harper *et al.*, 1999; Antzutkin, 2004). Synthetic 17–40 K28C dimers linked through a disulfide bond undergo changes in β -sheet in their FTIR spectra and seed protofibril and fibril formation (Schmechel *et al.*, 2003). An antibody raised against the dimeric species, MX-02, displays enhanced sensitivity for SDS-stable dimers of the wild-type peptide. This reactivity is similar to that of 4G8, which has been reported to preferentially label SDS-stable dimers on blots (Enya *et al.*, 1999). Elevated levels of the dimer species relative to monomer are found in AD brain compared to control brain (Schmechel *et al.*, 2003).

Higher order oligomeric species of A β , which have been recognized by EM and AFM, display yet other features. Spherical structures of 5 nm z-height are formed *in vitro* from A β (1–42) at low temperature (Chromy *et al.*, 2003; Stine *et al.*, 2003). A β -derived diffusible ligands (ADDLs) and other soluble multimeric A β species produce toxic effects at low concentration in a variety of cellular and physiological systems (El-Agnaf *et al.*, 2000; Walsh *et al.*, 2002; Caughey and Lansbury, 2003; Hoshi *et al.*, 2003; Kim *et al.*, 2003). These seem to be similar if not identical to species that appear early during fibril formation observed by a number of groups. When ADDLs are used to immunize rabbits (Lambert *et al.*, 2001), the resulting polyclonal antibodies M93 and 94 recognize SDS-stable 4–24-mer oligomers, depending on conditions of formation, in synthetic ADDL preparations, and primarily a 12-mer in AD brain. The oligomers are not detected in the control brain. Monomers do not react with these antibodies, although M93 and M94 crossreact with fibrils (Gong *et al.*, 2003). The nature of the crossreactivity has not been reported.

A different approach to generating oligomer-specific antibodies was based on the idea that the spherical oligomers were micellar assemblies. By attaching β (1–40) V40C to colloidal gold particles through the C-terminal thiol, A β micellar mimics were produced, that, because of the local high concentration of the peptide, formed a β -sheet structure on the surface of the colloidal particles (Kayed *et al.*, 2003). This conjugate elicited antibodies (M16) in rabbits that recognized authentic oligomeric A β (1–40) and A β (1–42), but not the monomeric or fibrillar peptides. The smallest oligomers that reacted with this antibody were 40 kDa. Dot blots detected oligomers in AD but not the control brain. Immunohistochemistry revealed characteristic patterns of oligomer reactivity around diffuse deposits, distinct from ThS-positive plaques. Oligomers could be detected within processes and synapses of Tg2576 hA β PP mouse brain, cultured neurons from the transgenic mouse brain, and in human AD brain (Takahashi *et al.*, 2004).

Furthermore, oligomers, but not monomers or fibrils, of the amyloidogenic proteins amylin (IAPP), α -synuclein, lysozyme, human insulin, prion 106–126, and polyglutamine all reacted strongly with the oligomer-specific M16 antibody and the Fab fragments derived from it. The antibody blocked cellular toxicity of oligomers of these proteins, but not the toxicity of the fibrillar forms. Thus, this antibody recognizes a general structural characteristic of amyloid oligomers that have been described for all of these proteins.

It is difficult to know with certainty what to make of the apparent specificities of antibodies raised against different conformational and organizational forms of A β . The techniques used to determine selectivity (dot blot, SDS-PAGE, Western blot, ELISA) among the different forms are not comparable in sensitivity. No head-to-head comparisons have yet been published.

7. Abbreviations

A β	Amyloid β peptide
A β PP	Amyloid β precursor protein
AFM	Atomic force microscopy
ADDL	A β -derived diffusible ligand
AD	Alzheimer's disease
ApoE	Apolipoprotein E
EM	Electron microscopy
bFGF	Basic fibroblast growth factor
FTIR	Fourier transform infrared
IAPP	Amylin
NFT	neurofibrillary tangle
NMR	Nuclear magnetic resonance
PET	Positron emission tomography
SAP	Serum amyloid P protein
SPECT	Single photon emission computed tomography
ThS	Thioflavine S
ThT	Thioflavin T
TTR	Transthyretin

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References

- Alessenko, A.V., Bugrova, A.E., and Dudnik, L.B. (2004). Connection of lipid peroxide oxidation with the sphingomyelin pathway in the development of Alzheimer's disease. *Biochem. Soc. Trans.* 32:144–146.
- Ando, Y., Haraoka, K., Terazaki, H., Tanoue, Y., Ishikawa, K., Katsuragi, S., Nakamura, M., Sun, X., Nakagawa, K., Sasamoto, K., Takesako, K., Ishizaki, T., Sasaki, Y., and Doh-Ura, K. (2003). A novel tool for detecting amyloid deposits in systemic amyloidosis in vitro and in vivo. *Lab. Invest.* 83:1751–1759.
- Ansari-Lari, M.A., and Ali, S.Z. (2004). Fine-needle aspiration of abdominal fat pad for amyloid detection: a clinically useful test? *Diagn. Cytopathol.* 30:178–181.
- Antzutkin, O.N. (2004). Amyloidosis of alzheimer's abeta peptides: solid-state nuclear magnetic resonance, electron paramagnetic resonance, transmission electron microscopy, scanning transmission electron microscopy and atomic force microscopy studies. *Magn. Reson. Chem.* 42:231–246.
- Bacskaï, B.J., Hickey, G.A., Skoch, J., Kajdasz, S.T., Wang, Y., Huang, G.F., Mathis, C.A., Klunk, W.E., and Hyman, B.T. (2003). Four-dimensional multiphoton imaging of brain entry, amyloid binding, and clearance of an amyloid-beta ligand in transgenic mice. *Proc. Natl. Acad. Sci. USA* 100:12462–12467.
- Bard, F., Cannon, C., Barbour, R., Burke, R.L., Games, D., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson Wood, K., Khan, K., Kholodenko, D., Lee, M., Lieberburg, I., Motter, R., Nguyen, M., Soriano, F., Vasquez, N., Weiss, K., Welch, B., Seubert, P., Schenk, D., and Yednock, T. (2000). Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. *Nat. Med.* 6:916–919.

- Bard, F., Barbour, R., Cannon, C., Carretto, R., Fox, M., Games, D., Guido, T., Hoenow, K., Hu, K., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, C., Lee, M., Motter, R., Nguyen, M., Reed, A., Schenk, D., Tang, P., Vasquez, N., Seubert, P., and Yednock, T. (2003). Epitope and isotype specificities of antibodies to beta-amyloid peptide for protection against Alzheimer's disease-like neuropathology. *Proc. Natl. Acad. Sci. USA* 100:2023–2028.
- Baskakov, I.V., Legname, G., Baldwin, M.A., Prusiner, S.B., and Cohen, F.E. (2002). Pathway complexity of prion protein assembly into amyloid. *J. Biol. Chem.* 277:21140–21148.
- Bieschke, J., Giese, A., Schulz Schaeffer, W., Zerr, I., Poser, S., Eigen, M., and Kretzschmar, H. (2000). Ultrasensitive detection of pathological prion protein aggregates by dual-color scanning for intensely fluorescent targets. *Proc. Natl. Acad. Sci. USA* 97:5468–5473.
- Blass, J.P. (2002). Potential for a specific neuroradiologic diagnosis of Alzheimer's disease. *J. Nucl. Med.* 43:1052–1053.
- Brandan, E., and Inestrosa, N.C. (1993). Extracellular matrix components and amyloid in neuritic plaques of Alzheimer's disease. *Gen. Pharmacol.* 24:1063–1068.
- Cai, L., Chin, F.T., Pike, V.W., Toyama, H., Liow, J.S., Zoghbi, S.S., Modell, K., Briard, E., Shetty, H.U., Sinclair, K., Donohue, S., Tipre, D., Kung, M.P., Dagostin, C., Widdowson, D.A., Green, M., Gao, W., Herman, M.M., Ichise, M., and Innis, R.B. (2004). Synthesis and evaluation of two (18)f-labeled 6-iodo-2-(4'-n,n-dimethylamino)phenyli midazo[1,2-a]pyridine derivatives as prospective radioligands for beta-amyloid in Alzheimer's disease. *J. Med. Chem.* 47:2208–2218.
- Cannon, M.J., Williams, A.D., Wetzel, R., and Myszka, D.G. (2004). Kinetic analysis of beta-amyloid fibril elongation. *Anal. Biochem.* 328:67–75.
- Carter, D.B., and Chou, K.C. (1998). A model for structure-dependent binding of Congo Red to Alzheimer beta-amyloid fibrils. *Neurobiol. Aging* 19:37–40.
- Caughey, B., and Lansbury, P.T., Jr. (2003). Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders. *Annu. Rev. Neurosci.*
- Chauhan, N.B., and Siegel, G.J. (2003). Intracerebroventricular passive immunization with anti-abeta antibody in Tg2576. *J. Neurosci. Res.* 74:142–147.
- Chen, S., Berthelie, V., Hamilton, J.B., O'Nuallain, B., and Wetzel, R. (2002). Amyloid-like features of polyglutamine aggregates and their assembly kinetics. *Biochemistry* 41:7391–7399.
- Chien, P., and Weissman, J.S. (2001). Conformational diversity in a yeast prion dictates its seeding specificity. *Nature* 410:223–227.
- Chien, P., DePace, A.H., Collins, S.R., and Weissman, J.S. (2003). Generation of prion transmission barriers by mutational control of amyloid conformations. *Nature* 424:948–951.
- Choo, L.P., Wetzel, D.L., Halliday, W.C., Jackson, M., LeVine, S.M., and Mantsch, H.H. (1996). In situ characterization of beta-amyloid in Alzheimer's diseased tissue by synchrotron fourier transform infrared microspectroscopy. *Biophys. J.* 71:1672–1679.
- Christen, Y. (2000). Oxidative stress and alzheimer disease. *Am. J. Clin. Nutr.* 71:621s–629s.
- Chromy, B.A., Nowak, R.J., Lambert, M.P., Viola, K.L., Chang, L., Velasco, P.T., Jones, B.W., Fernandez, S.J., Lacor, P.N., Horowitz, P., Finch, C.E., Krafft, G.A., and Klein, W.L. (2003). Self-assembly of Abeta(1–42) into globular neurotoxins. *Biochemistry* 42:12749–12760.
- Conway, K.A., Lee, S.J., Rochet, J.C., Ding, T.T., Williamson, R.E., and Lansbury, P.T. (2000). Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy. *Proc. Natl. Acad. Sci. USA* 97:571–576.
- Crystal, A.S., Giasson, B.I., Crowe, A., Kung, M.P., Zhuang, Z.P., Trojanowski, J.Q., and Lee, V.M. (2003). A comparison of amyloid fibrillogenesis using the novel fluorescent compound K114. *J. Neurochem.* 86:1359–1368.
- Cummings, B.J., Su, J.H., and Cotman, C.W. (1993). Neuritic involvement within bFGF immunopositive plaques of Alzheimer's disease. *Exp. Neurol.* 124:315–325.
- El-Agnaf, O.M., Mahil, D.S., Patel, B.P., and Austen, B.M. (2000). Oligomerization and toxicity of beta-amyloid-42 implicated in Alzheimer's disease. *Biochem. Biophys. Res. Commun.* 273:1003–1007.
- Enya, M., Morishima Kawashima, M., Yoshimura, M., Shinkai, Y., Kusui, K., Khan, K., Games, D., Schenk, D., Sugihara, S., Yamaguchi, H., and Ihara, Y. (1999). Appearance of sodium dodecyl sulfate-stable amyloid beta-protein (abeta) dimer in the cortex during aging. *Am. J. Pathol.* 154:271–279.
- Esler, W.P., Stimson, E.R., Ghilardi, J.R., Felix, A.M., Lu, Y.-A., Vinters, H.V., Mantyh, P.J., and Maggio, J.E. (1997). Ab deposition inhibitor screen using synthetic amyloid. *Nature Biotechnology* 15:258–263.
- Esler, W.P., Stimson, E.R., Mantyh, P.W., and Maggio, J.E. (1999). Deposition of soluble amyloid-beta onto amyloid templates: with application for the identification of amyloid fibril extension inhibitors. *Methods Enzymol.* 309:350–374.

- Esler, W.P., Felix, A.M., Stimson, E.R., Lachenmann, M.J., Ghilardi, J.R., Lu, Y.A., Vinters, H.V., Mantyh, P.W., Lee, J.P., and Maggio, J.E. (2000a). Activation barriers to structural transition determine deposition rates of Alzheimer's disease β amyloid. *J. Struct. Biol.* 130:174–183.
- Esler, W.P., Stimson, E.R., Jennings, J.M., Vinters, H.V., Ghilardi, J.R., Lee, J.P., Mantyh, P.W., and Maggio, J.E. (2000b). Alzheimer's disease amyloid propagation by a template-dependent dock-lock mechanism. *Biochemistry* 39:6288–6295.
- Ferrer, I., Boada Rovira, M., Sanchez Guerra, M.L., Rey, M.J., and Costa-Jussa, F. (2004). Neuropathology and pathogenesis of encephalitis following amyloid- β immunization in Alzheimer's disease. *Brain Pathol.* 14: 11–20.
- Frenkel, D., Dewachter, I., Van Leuven, F., and Solomon, B. (2003). Reduction of β -amyloid plaques in brain of transgenic mouse model of Alzheimer's disease by EFRH phage immunization. *Vaccine* 21:1060–1065.
- Friedland, R.P., Shi, J., Lamanna, J.C., Smith, M.A., and Perry, G. (2000). Prospects for noninvasive imaging of brain amyloid β in Alzheimer's disease. *Ann. N. Y. Acad. Sci.* 903:123–128.
- Games, D., Bard, F., Grajeda, H., Guido, T., Khan, K., Soriano, F., Vasquez, N., Wehner, N., Johnson Wood, K., Yednock, T., Seubert, P., and Schenk, D. (2000). Prevention and reduction of ad-type pathology in PDAPP mice immunized with a β 1–42. *Ann. N. Y. Acad. Sci.* 920:274–284.
- Gibson, G., Gunasekera, N., Lee, M., Lelyveld, V., El-Agnaf, O.M., Wright, A., and Austen, B. (2004). Oligomerization and neurotoxicity of the amyloid β peptide implicated in familial Danish dementia. *J. Neurochem.* 88: 281–290.
- Goldsbury, C., Kistler, J., Aebi, U., Arvinte, T., and Cooper, G.J. (1999). Watching amyloid fibrils grow by time-lapse atomic force microscopy. *J. Mol. Biol.* 285:33–39.
- Gong, Y., Chang, L., Viola, K.L., Lacor, P.N., Lambert, M.P., Finch, C.E., Krafft, G.A., and Klein, W.L. (2003). Alzheimer's disease-affected brain: presence of oligomeric β ligands (ADDLS) suggests a molecular basis for reversible memory loss. *Proc. Natl. Acad. Sci. USA* 100:10417–10422.
- Green, J.D., Goldsberry, C., Kistler, J., Cooper, G.S., and Aebi, U. (2004). Human amylin oligomer growth and fibril elongation define two distinct phases in amyloid formation. *J. Biol. Chem.*
- Harper, J.D., Wong, S.S., Lieber, C.M., and Lansbury, P.T. (1997). Observation of metastable β amyloid protofibrils by atomic force microscopy. *Chem. Biol.* 4:119–125.
- Harper, J.D., Wong, S.S., Lieber, C.M., and Lansbury, P.T., Jr. (1999). Assembly of a β amyloid protofibril: an in vitro model for a possible early event in Alzheimer's disease. *Biochemistry* 38:8972–8980.
- Hock, C., Konietzko, U., Streffer, J.R., Tracy, J., Signorell, A., Muller-Tillmanns, B., Lemke, U., Henke, K., Moritz, E., Garcia, E., Wollmer, M.A., Umbrecht, D., de Quervain, D.J., Hofmann, M., Muddalena, A., Papassotiropoulos, A., and Nitsch, R.M. (2003). Antibodies against β -amyloid slow cognitive decline in Alzheimer's disease. *Neuron* 38:547–554.
- Hoshi, M., Sato, M., Matsumoto, S., Noguchi, A., Yasutake, K., Yoshida, N., and Sato, K. (2003). Spherical aggregates of β -amyloid (amylospheroid) show high neurotoxicity and activate tau protein kinase glycogen synthase kinase-3 β . *Proc. Natl. Acad. Sci. USA* 100:6370–6375.
- Huang, X., Moir, R.D., Tanzi, R.E., Bush, A.I., and Rogers, J.T. (2004). Redox-active metals, oxidative stress, and Alzheimer's disease pathology. *Ann. N. Y. Acad. Sci.* 1012:153–163.
- Ionescu-Zanetti, C., Khurana, R., Gillespie, J.R., Petrick, J.S., Trabachino, L.C., Minert, L.J., Carter, S.A., and Fink, A.L. (1999). Monitoring the assembly of Ig light-chain amyloid fibrils by atomic force microscopy. *Proc. Natl. Acad. Sci. USA* 96:13175–13179.
- Jicha, G.A., Bowser, R., Kazam, I.G., and Davies, P. (1997a). Alz-50 and MC-1, a new monoclonal antibody raised to paired helical filaments, recognize conformational epitopes on recombinant tau. *J. Neurosci. Res.* 48:128–132.
- Jicha, G.A., Lane, E., Vincent, I., Otvos, L., Jr., Hoffmann, R., and Davies, P. (1997b). A conformation- and phosphorylation-dependent antibody recognizing the paired helical filaments of Alzheimer's disease. *J. Neurochem.* 69:2087–2095.
- Jicha, G.A., Rockwood, J.M., Berenfeld, B., Hutton, M., and Davies, P. (1999). Altered conformation of recombinant frontotemporal dementia-17 mutant tau proteins. *Neurosci. Lett.* 260:153–156.
- Jin, L.W., Claborn, K.A., Kurimoto, M., Geday, M.A., Maezawa, I., Sohraby, F., Estrada, M., Kaminsky, W., and Kahr, B. (2003). Imaging linear birefringence and dichroism in cerebral amyloid pathologies. *Proc. Natl. Acad. Sci. USA* 100:15294–15298.
- Kad, N.M., Myers, S.L., Smith, D.P., Alastair Smith, D., Radford, S.E., and Thomson, N.H. (2003). Hierarchical assembly of β (2)-microglobulin amyloid in vitro revealed by atomic force microscopy. *J. Mol. Biol.* 330:785–797.

- Kang, D.E., Pietrzik, C.U., Baum, L., Chevallier, N., Merriam, D.E., Kounnas, M.Z., Wagner, S.L., Troncoso, J.C., Kawas, C.H., Katzman, R., and Koo, E.H. (2000). Modulation of amyloid beta-protein clearance and Alzheimer's disease susceptibility by the LDL receptor-related protein pathway. *J. Clin. Invest.* 106:1159–1166.
- Kayed, R., Bernhagen, J., Greenfield, N., Sweimeh, K., Brunner, H., Voelter, W., and Kapurniotu, A. (1999). Conformational transitions of islet amyloid polypeptide (IAPP) in amyloid formation in vitro. *J. Mol. Biol.* 287:781–796.
- Kayed, R., Head, E., Thompson, J.L., McIntire, T.M., Milton, S.C., Cotman, C.W., and Glabe, C.G. (2003). Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science* 300:486–489.
- Kelenyi, G. (1967). Thioflavin S fluoresces and Congo Red anisotropic stainings in the histologic demonstration of amyloid. *Acta Neuropathol. (Berl.)* 7:336–348.
- Kheterpal, I., Zhou, S., Cook, K.D., and Wetzel, R. (2000). Abeta amyloid fibrils possess a core structure highly resistant to hydrogen exchange. *Proc. Natl. Acad. Sci. USA* 97:13597–13601.
- Kheterpal, I., Williams, A., Murphy, C., Bledsoe, B., and Wetzel, R. (2001). Structural features of the abeta amyloid fibril elucidated by limited proteolysis. *Biochemistry* 40:11757–11767.
- Kheterpal, I., Lashuel, H.A., Hartley, D.M., Walz, T., Lansbury, P.T., Jr. and Wetzel, R. (2003a). Abeta protofibrils possess a stable core structure resistant to hydrogen exchange. *Biochemistry* 42:14092–14098.
- Kheterpal, I., Wetzel, R., and Cook, K.D. (2003b). Enhanced correction methods for hydrogen exchange-mass spectrometric studies of amyloid fibrils. *Protein Sci.* 12:635–643.
- Khurana, R., Ionescu-Zanetti, C., Pope, M., Li, J., Nielson, L., Ramirez-Alvarado, M., Regan, L., Fink, A.L., and Carter, S.A. (2003). A general model for amyloid fibril assembly based on morphological studies using atomic force microscopy. *Biophys. J.* 85:1135–1144.
- Kim, H.J., Chae, S.C., Lee, D.K., Chromy, B., Lee, S.C., Park, Y.C., Klein, W.L., Krafft, G.A., and Hong, S.T. (2003). Selective neuronal degeneration induced by soluble oligomeric amyloid beta protein. *FASEB J.* 17:118–120.
- Kirkitadze, M.D., Condron, M.M., and Teplow, D.B. (2001). Identification and characterization of key kinetic intermediates in amyloid beta-protein fibrillogenesis. *J. Mol. Biol.* 312:1103–1119.
- Kisilevsky, R., and Fraser, P. (1996). Proteoglycans and amyloid fibrillogenesis. *Ciba Found. Symp.* 199:58–67.
- Kisilevsky, R., Lyon, A.W., and Young, I.D. (1992). A critical analysis of postulated pathogenetic mechanisms in amyloidogenesis. *Crit. Rev. Clin. Lab. Sci.* 29:59–82.
- Klunk, W.E., Debnath, M.L., and Pettegrew, J.W. (1994). Development of small molecule probes for the beta-amyloid protein of Alzheimer's disease. *Neurobiol. Aging* 15:691–698.
- Klunk, W.E., Jacob, R.F., and Mason, R.P. (1999). Quantifying amyloid by Congo Red spectral shift assay. *Methods Enzymol.* 309:285–305.
- Klunk, W.E., Engler, H., Nordberg, A., Wang, Y., Blomqvist, G., Holt, D.P., Bergstrom, M., Savitcheva, I., Huang, G.F., Estrada, S., Aussen, B., Debnath, M.L., Barletta, J., Price, J.C., Sandell, J., Lopresti, B.J., Wall, A., Koivisto, P., Antoni, G., Mathis, C.A., and Langstrom, B. (2004). Imaging brain amyloid in Alzheimer's disease with Pittsburgh compound-B. *Ann. Neurol.* 55:306–319.
- Korth, C., Streit, P., and Oesch, B. (1999). Monoclonal antibodies specific for the native, disease-associated isoform of the prion protein. *Methods Enzymol.* 309:106–122.
- Ksiezak-Reding, H., Leibowitz, R.L., Bowser, R., and Davies, P. (1995). Binding of Alz 50 depends on phe8 in tau synthetic peptides and varies between native and denatured tau proteins. *Brain Res.* 697:63–75.
- Kung, M.P., Skovronsky, D.M., Hou, C., Zhuang, Z.P., Gur, T.L., Zhang, B., Trojanowski, J.Q., Lee, V.M., and Kung, H.F. (2003). Detection of amyloid plaques by radioligands for abeta40 and abeta42: potential imaging agents in Alzheimer's patients. *J. Mol. Neurosci.* 20:15–24.
- Kurihara, A., and Pardridge, W.M. (2000). Abeta(1–40) peptide radiopharmaceuticals for brain amyloid imaging: (111)In chelation, conjugation to poly(ethylene glycol)-biotin linkers, and autoradiography with Alzheimer's disease brain sections. *Bioconjug. Chem.* 11:380–386.
- Ladu, M.J., Reardon, C., Van Eldik, L., Fagan, A.M., Bu, G., Holtzman, D., and Getz, G.S. (2000). Lipoproteins in the central nervous system. *Ann. N. Y. Acad. Sci.* 903:167–175.
- LaDu, M.J., Shah, J.A., Reardon, C.A., Getz, G.S., Bu, G., Hu, J., Guo, L., and van Eldik, L.J. (2000). Apolipoprotein E receptors mediate the effects of beta-amyloid on astrocyte cultures. *J. Biol. Chem.* 275:33974–33980.
- Lambert, M.P., Viola, K.L., Chromy, B.A., Chang, L., Morgan, T.E., Yu, J., Venton, D.L., Krafft, G.A., Finch, C.E., and Klein, W.L. (2001). Vaccination with soluble abeta oligomers generates toxicity-neutralizing antibodies. *J. Neurochem.* 79:595–605.
- Leclerc, E., Peretz, D., Ball, H., Solforosi, L., Legname, G., Safar, J., Serban, A., Prusiner, S.B., Burton, D.R., and Williamson, R.A. (2003). Conformation of prp(c) on the cell surface as probed by antibodies. *J. Mol. Biol.* 326:475–483.

- Lee, H.J., Zhang, Y., Zhu, C., Duff, K., and Pardridge, W.M. (2002). Imaging brain amyloid of Alzheimer disease in vivo in transgenic mice with an abeta peptide radiopharmaceutical. *J. Cereb. Blood Flow Metab.* 22:223–231.
- Lee, V.M. (2002). Amyloid binding ligands as Alzheimer's disease therapies. *Neurobiol. Aging* 23:1039–1042.
- Legleiter, J., and Kowalewski, T. (2003). Atomic force microscopy of beta-amyloid: static and dynamic studies of nano-structure and its formation. *Methods Mol. Biol.* 242:349–364.
- LeVine, H., III. (1993). Thioflavine T interaction with synthetic Alzheimer's disease beta-amyloid peptides: detection of amyloid aggregation in solution. *Protein Sci.* 2:404–410.
- LeVine, H., III. (1995). Thioflavine T interaction with amyloid b-sheet structures. *Amyloid Int. J. Exp. Clin. Invest.* 2:1–6.
- LeVine, H., 3rd. (1999). Quantification of beta-sheet amyloid fibril structures with Thioflavin T. *Methods Enzymol.* 309:274–284.
- LeVine, H., 3rd. (2000). ¹²⁵I-labeled apoE binds competitively to beta(1–40) fibrils with pathological chaperone proteins. *Amyloid* 7:83–89.
- LeVine, H., III. (2002). 4,4'(-)-dianilino-1,1'(-)-binaphthyl-5,5'(-)-disulfonate: report on non-beta-sheet conformers of Alzheimer's peptide beta(1–40). *Arch. Biochem. Biophys.* 404:106–115.
- LeVine, H., 3rd. (2003). Y10W beta(1–40) fluorescence reflects epitope exposure in conformers of Alzheimer's beta-peptide. *Arch. Biochem. Biophys.* 417:112–122.
- Maggio, J.E., Stimson, E.R., Ghilardi, J.R., Allen, C.J., Dahl, C.E., Whitcomb, D.C., Vignon, S.R., Vinters, H.V., Labenski, M.E., and Mantyh, P.W. (1992). Reversible *in vitro* growth of Alzheimer disease b-amyloid plaques by deposition of labeled amyloid peptide. *Proc. Natl. Acad. Sci. USA* 89:5462–5466.
- Matsunaga, Y., Peretz, D., Williamson, A., Burton, D., Mehlhorn, I., Groth, D., Cohen, F.E., Prusiner, S.B., and Baldwin, M.A. (2001). Cryptic epitopes in N-terminally truncated prion protein are exposed in the full-length molecule: dependence of conformation on pH. *Proteins* 44:110–118.
- Matsunaga, Y., Saito, N., Fujii, A., Yokotani, J., Takakura, T., Nishimura, T., Esaki, H., and Yamada, T. (2002). A pH-dependent conformational transition of abeta peptide and physicochemical properties of the conformers in the glial cell. *Biochem. J.* 361:547–556.
- McGeer, E.G., and McGeer, P.L. (2003). Inflammatory processes in Alzheimer's disease. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 27:741–749.
- McGeer, P.L., and McGeer, E.G. (2002). Innate immunity, local inflammation, and degenerative disease. *Sci. Aging Knowledge Environ.* 2002:re3.
- McGeer, P.L., Klegeris, A., Walker, D.G., Yasuhara, O., and McGeer, E.G. (1994). Pathological proteins in senile plaques. *Tohoku J. Exp. Med.* 174:269–277.
- Miller, D.L., Currie, J.R., Mehta, P.D., Potempska, A., Hwang, Y.W., and Wegiel, J. (2003). Humoral immune response to fibrillar beta-amyloid peptide. *Biochemistry* 42:11682–11692.
- Miyata, T., Ueda, Y., Saito, A., and Kurokawa, K. (2000). Carbonyl stress' and dialysis-related amyloidosis. *Nephrol. Dial. Transplant.* 15:125–128.
- Modler, A.J., Gast, K., Lutsch, G., and Damaschun, G. (2003). Assembly of amyloid protofibrils via critical oligomers—a novel pathway of amyloid formation. *J. Mol. Biol.* 325:135–148.
- Naiki, H., Higuchi, K., Hosokawa, M., and Takeda, T. (1989). Fluorometric determination of amyloid fibrils in vitro using the fluorescent dye, Thioflavin T. *Anal. Biochem.* 177:244–249.
- Nicoll, J.A., Wilkinson, D., Holmes, C., Steart, P., Markham, H., and Weller, R.O. (2003). Neuropathology of human alzheimer disease after immunization with amyloid-beta peptide: a case report. *Nat. Med.* 9:448–452.
- Nordstedt, C., Naslund, J., Tjernberg, L.O., Karlstrom, A.R., Thyberg, J., and Terenius, L. (1994). The Alzheimer abeta peptide develops protease resistance in association with its polymerization into fibrils. *J. Biol. Chem.* 269:30773–30776.
- Olsen, K.E., Sletten, K., and Westermark, P. (1999). The use of subcutaneous fat tissue for amyloid typing by enzyme-linked immunosorbent assay. *Am. J. Clin. Pathol.* 111:355–362.
- O'Nuallain, B., and Wetzel, R. (2002). Conformational Abs recognizing a generic amyloid fibril epitope. *Proc. Natl. Acad. Sci. USA* 99:1485–1490.
- O'Nuallain, B., Williams, A.D., Westermark, P., and Wetzel, R. (2004). Seeding specificity in amyloid growth induced by heterologous fibrils. *J. Biol. Chem.* 279:17490–17499.
- Orgogozo, J.M., Gilman, S., Dartigues, J.F., Laurent, B., Puel, M., Kirby, L.C., Jouanny, P., Dubois, B., Eisner, L., Flitman, S., Michel, B.F., Boada, M., Frank, A., and Hock, C. (2003). Subacute meningoencephalitis in a subset of patients with AD after abeta42 immunization. *Neurology* 61:46–54.
- Pepys, M.B., Herbert, J., Hutchinson, W.L., Tennent, G.A., Lachmann, H.J., Gallimore, J.R., Lovat, L.B., Bartfai, T., Alanine, A., Hertel, C., Hoffmann, T., Jakob-Roetne, R., Norcross, R.D., Kemp, J.A., Yamamura, K., Suzuki, M.,

- Taylor, G.W., Murray, S., Thompson, D., Purvis, A., Kolstoe, S., Wood, S.P., and Hawkins, P.N. (2002). Targeted pharmacological depletion of serum amyloid P component for treatment of human amyloidosis. *Nature* 417:254–259.
- Peretz, D., Scott, M.R., Groth, D., Williamson, R.A., Burton, D.R., Cohen, F.E., and Prusiner, S.B. (2001). Strain-specified relative conformational stability of the scrapie prion protein. *Protein Sci.* 10:854–863.
- Peretz, D., Williamson, R.A., Legname, G., Matsunaga, Y., Vergara, J., Burton, D.R., DeArmond, S.J., Prusiner, S.B., and Scott, M.R. (2002). A change in the conformation of prions accompanies the emergence of a new prion strain. *Neuron* 34:921–932.
- Perez, M.K., Paulson, H.L., and Pittman, R.N. (1999). Ataxin-3 with an altered conformation that exposes the polyglutamine domain is associated with the nuclear matrix. *Hum. Mol. Genet.* 8:2377–2385.
- Perutz, M.F., Finch, J.T., Berriman, J., and Lesk, A. (2002). Amyloid fibers are water-filled nanotubes. *Proc. Natl. Acad. Sci. USA* 99:5591–5595.
- Pitschke, M., Prior, R., Haupt, M., and Riesner, D. (1998). Detection of single amyloid beta-protein aggregates in the cerebrospinal fluid of Alzheimer's patients by fluorescence correlation spectroscopy [see comments]. *Nat. Med.* 4:832–834.
- Poduslo, J.F., Wengenack, T.M., Curran, G.L., Wisniewski, T., Sigurdsson, E.M., Macura, S.I., Borowski, B.J., and Jack, C.R., Jr. (2002). Molecular targeting of Alzheimer's amyloid plaques for contrast-enhanced magnetic resonance imaging. *Neurobiol. Dis.* 11:315–329.
- Poirier, J. (2000). Apolipoprotein E and Alzheimer's disease. A role in amyloid catabolism. *Ann. N. Y. Acad. Sci.* 924:81–90.
- Poirier, J., Davignon, J., Bouthillier, D., Kogan, S., Bertrand, P., and Gauthier, S. (1993). Apolipoprotein E polymorphism and Alzheimer's disease. *Lancet* 342:697–699.
- Poirier, J., Delisle, M.C., Quirion, R., Aubert, I., Farlow, M., Lahiri, D., Hui, S., Bertrand, P., Nalbantoglu, J., Gilfix, B.M., and Gauthier, S. (1995). Apolipoprotein ε4 allele as a predictor of cholinergic deficits and treatment outcome in Alzheimer disease. *Proc. Natl. Acad. Sci. USA* 92:12260–12264.
- Scheibel, T., Bloom, J., and Lindquist, S.L. (2004). The elongation of yeast prion fibers involves separable steps of association and conversion. *Proc. Natl. Acad. Sci. USA* 101:2287–2292.
- Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson Wood, K., Khan, K., Kholodenko, D., Lee, M., Liao, Z., Lieberburg, I., Motter, R., Mutter, L., Soriano, F., Shopp, G., Vasquez, N., Vandevent, C., Walker, S., Wogulis, M., Yednock, T., Games, D., and Seubert, P. (1999). Immunization with amyloid-beta attenuates alzheimer-disease-like pathology in the pdapp mouse. *Nature* 400:173–177.
- Schmechel, A., Zentgraf, H., Scheuermann, S., Fritz, G., Pipkorn, R., Reed, J., Beyreuther, K., Bayer, T.A., and Multhaup, G. (2003). Alzheimer's beta-amyloid homodimers facilitate abeta fibrillization and the generation of conformational antibodies. *J. Biol. Chem.*
- Schutkowski, M., Bernhardt, A., Zhou, X.Z., Shen, M., Reimer, U., Rahfeld, J.U., Lu, K.P., and Fischer, G. (1998). Role of phosphorylation in determining the backbone dynamics of the serine/threonine-proline motif and Pin1 substrate recognition. *Biochemistry* 37:5566–5575.
- Serpell, L.C., Sunde, M., Benson, M.D., Tennent, G.A., Pepys, M.B., and Fraser, P.E. (2000). The protofilament substructure of amyloid fibrils. *J. Mol. Biol.* 300:1033–1039.
- Shi, J., Perry, G., Berridge, M.S., Aliev, G., Siedlak, S.L., Smith, M.A., LaManna, J.C., and Friedland, R.P. (2002). Labeling of cerebral amyloid beta deposits in vivo using intranasal basic fibroblast growth factor and serum amyloid P component in mice. *J. Nucl. Med.* 43:1044–1051.
- Shoghi-Jadid, K., Small, G.W., Agdeppa, E.D., Kepe, V., Ercoli, L.M., Siddarth, P., Read, S., Satyamurthy, N., Petric, A., Huang, S.C., and Barrio, J.R. (2002). Localization of neurofibrillary tangles and beta-amyloid plaques in the brains of living patients with Alzheimer disease. *Am. J. Geriatr. Psychiatry* 10:24–35.
- Sigurdsson, E.M., Scholtzova, H., Mehta, P.D., Frangione, B., and Wisniewski, T. (2001). Immunization with a nontoxic/nonfibrillar amyloid-beta homologous peptide reduces Alzheimer's disease-associated pathology in transgenic mice. *Am. J. Pathol.* 159:439–447.
- Sipe, J.D., and Cohen, A.S. (2000). Review: history of the amyloid fibril. *J. Struct. Biol.* 130:88–98.
- Skovronsky, D.M., Zhang, B., Kung, M.P., Kung, H.F., Trojanowski, J.Q., and Lee, V.M. (2000). In vivo detection of amyloid plaques in a mouse model of Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* 97:7609–7614.
- Stine, W.B., Jr., Snyder, S.W., Lador, U.S., Wade, W.S., Miller, M.F., Perun, T.J., Holzman, T.F., and Krafft, G.A. (1996). The nanometer-scale structure of amyloid-beta visualized by atomic force microscopy. *J. Protein Chem.* 15:193–203.
- Stine, W.B., Jr., Dahlgren, K.N., Krafft, G.A., and LaDu, M.J. (2003). In vitro characterization of conditions for amyloid-beta peptide oligomerization and fibrillogenesis. *J. Biol. Chem.* 278:11612–11622.

- Styren, S.D., Hamilton, R.L., Styren, G.C., and Klunk, W.E. (2000). X-34, a fluorescent derivative of Congo Red: a novel histochemical stain for Alzheimer's disease pathology. *J. Histochem. Cytochem.* 48:1223–1232.
- Suemoto, T., Okamura, N., Shiomitsu, T., Suzuki, M., Shimadzu, H., Akatsu, H., Yamamoto, T., Kudo, Y., and Sawada, T. (2004). In vivo labeling of amyloid with BF-108. *Neurosci. Res.* 48:65–74.
- Sunde, M., Serpell, L.C., Bartlam, M., Fraser, P.E., Pepys, M.B., and Blake, C.C. (1997). Common core structure of amyloid fibrils by synchrotron x-ray diffraction. *J. Mol. Biol.* 273:729–739.
- Takahashi, R.H., Almeida, C.G., Kearney, P.F., Yu, F., Lin, M.T., Milner, T.A., and Gouras, G.K. (2004). Oligomerization of Alzheimer's beta-amyloid within processes and synapses of cultured neurons and brain. *J. Neurosci.* 24: 3592–3599.
- Trottier, Y., Lutz, Y., Stevanin, G., Imbert, G., Devys, D., Cancel, G., Saudou, F., Weber, C., David, G., Tora, L., *et al.* (1995). Polyglutamine expansion as a pathological epitope in Huntington's disease and four dominant cerebellar ataxias. *Nature* 378: 403–6.
- Tseng, B.P., Esler, W.P., Clish, C.B., Stimson, E.R., Ghilardi, J.R., Vinters, H.V., Mantyh, P.W., Lee, J.P., and Maggio, J.E. (1999). Deposition of monomeric, not oligomeric, abeta mediates growth of Alzheimer's disease amyloid plaques in human brain preparations. *Biochemistry* 38:10424–10431.
- Turnell, W.G., and Finch, J.T. (1992). Binding of the dye Congo Red to the amyloid protein pig insulin reveals a novel homology amongst amyloid-forming peptide sequences. *J. Mol. Biol.* 227:1205–1223.
- Tycko, R., and Ishii, Y. (2003). Constraints on supramolecular structure in amyloid fibrils from two-dimensional solid-state NMR spectroscopy with uniform isotopic labeling. *J. Am. Chem. Soc.* 125:6606–6607.
- Walker, L.C., Pahnke, J., Madauss, M., Vogelgesang, S., Pahnke, A., Herbst, E.W., Stauske, D., Walther, R., Kessler, C., and Warzok, R.W. (2000). Apolipoprotein ε4 promotes the early deposition of abeta42 and then abeta40 in the elderly. *Acta Neuropathol. (Berl.)* 100:36–42.
- Walsh, D.M., Klyubin, I., Fadeeva, J.V., Rowan, M.J., and Selkoe, D.J. (2002). Amyloid-beta oligomers: their production, toxicity and therapeutic inhibition. *Biochem. Soc. Trans.* 30:552–557.
- Wang, Z., Zhou, C., Wang, C., Wan, L., Fang, X., and Bai, C. (2003). AFM and STM study of beta-amyloid aggregation on graphite. *Ultramicroscopy* 97:73–79.
- Yamaguchi, K., Katou, H., Hoshino, M., Hasegawa, K., Naiki, H., and Goto, Y. (2004). Core and heterogeneity of beta(2)-microglobulin amyloid fibrils as revealed by H/D exchange. *J. Mol. Biol.* 338:559–571.

Three-Dimensional Structural Analysis of Amyloid Fibrils by Electron Microscopy

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1. Abstract

Amyloid fibrils are insoluble aggregates that result from the self-assembly of partially unfolded proteins. Regardless of the native structure of the precursor proteins, the predominant secondary structure in the fibrillar form is β -sheet. Proteins that form amyloid *in vivo* are associated with numerous diseases, including Alzheimer's, Parkinson's, and prion diseases. The three-dimensional structures of amyloid fibrils may provide valuable information on the misfolded protein conformations and on the pathways that lead to disease. The purpose of this chapter is to introduce electron microscopy as a tool for the structure determination of amyloid fibrils.

2. Introduction: Structural Studies

As the end point of an ordered misfolding and assembly/aggregation process, or as possible precursors of cytotoxic, smaller oligomeric species, amyloid fibrils are an interesting target for three-dimensional (3D) structural study. Their 3D structures may provide valuable information on the misfolded protein conformations and on the pathways that lead to disease. The purpose of the electron microscopy (EM) studies described in this chapter is to determine the 3D structure of amyloid fibrils. So far, this has only been possible for fibrils assembled *in vitro*, but the methods are also applicable to fibrils isolated *ex vivo*, if they are sufficiently well ordered. The resolution has so far been limited to the scale of protofilaments, rather than subunits or secondary structures, but advances in EM and computing hardware are likely to provide improvements. A characteristic of amyloid fibril structural studies is the availability of some detailed local information on the secondary structure of the polypeptide chain combined with a dearth of knowledge about how these structural elements assemble into the overall 3D structure of the fibril. As the resolution of cryo EM analysis improves, it may become possible to fill in the information gap between peptide conformation and protofilament assembly into fibrils.

3. Amyloid Fibril Structure and the Cross- β Fold

The typical EM appearance of amyloid fibrils is of long filaments, 60–120 Å in diameter, formed of laterally associated substructures called protofilaments (Shirahama and Cohen, 1967; Cohen *et al.*, 1982; Kirschner *et al.*, 1987; Goldsbury *et al.*, 1997; Serpell *et al.*, 2000). The term

protofibril is used to describe immature assemblies in early stages of fibril formation. The mature fibrils commonly exhibit a long-range helical twist. The variable morphology of the fibrils as well as the variable nature of the twist greatly complicate structural analysis. Both the number and arrangement of protofilaments vary within preparations, and the twist varies even within individual fibrils (Jiménez *et al.*, 1999, 2002). Figure 12.2-1 shows examples of different fibril morphologies found in a typical amyloid preparation.

Despite the absence of overall long-range order, X-ray fiber diffraction and cryo EM have provided direct evidence that the underlying structure of an amyloid fibril is a cross- β arrangement of β -strands, in which long, thin ribbons of β -sheet are aligned with the β -strands perpendicular to the fiber axis (Sunde *et al.*, 1997; Serpell and Smith, 2000) (Figure 12.2-2A and B). The characteristic 4.8 Å repeat along the fiber axis (meridional) corresponds to the distance between strands in a beta-sheet, and a diffuse ~ 10 Å reflection perpendicular to the fiber axis (equatorial) is attributed to the stacking of neighboring β -sheets. Supporting evidence for high β -structure content is provided by the circular dichroism and Fourier transform infrared spectra of amyloid fibrils. Recently, local

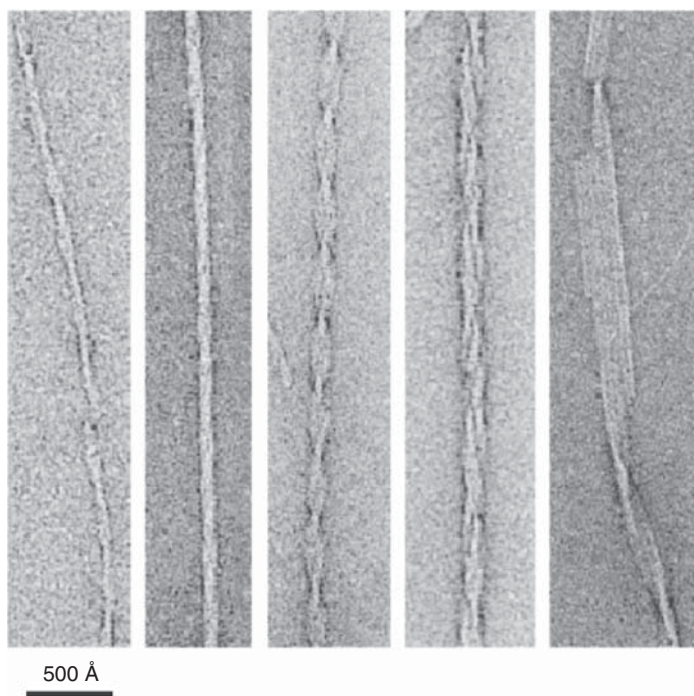
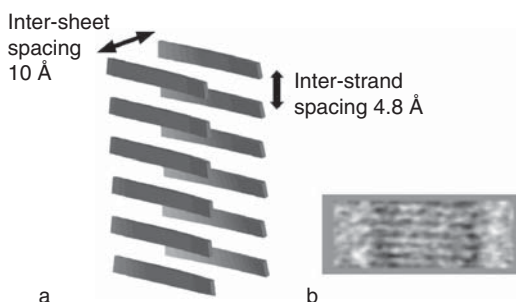


Figure 12.2-1. Negative-stain EM images of insulin amyloid fibrils from a single preparation, showing the diverse twists and numbers of protofilaments. (Reproduced from Jiménez *et al.*, 2002. Copyright 2002 from the National Academy of Sciences USA.)

Figure 12.2-2. The cross- β structure. (A) Cartoon of two ribbons of a β -sheet, showing the characteristic interstrand and intersheet spacings revealed by X-ray fiber diffraction. (b) Cryo EM image of an averaged segment of an A β fibril, showing the β -strand repeat. It is a remarkable achievement to image the 4.8-Å striations, which are visible because several β -sheets are aligned in register in these fibrils. (B) Reproduced from Serpell and Smith, 2000, by permission from Elsevier Press.



atomic structure information has been obtained by solid-state NMR (Jaroniec *et al.*, 2004; Tycko, 2004), providing evidence for both parallel and antiparallel β -sheet arrangements of different peptides. The range of this technique is limited to interatomic distances up to ~ 6.5 Å, making it difficult to extend the structural detail beyond a single strand or β -hairpin. X-ray and NMR methods involve averaging of fibrils in a heterogeneous bulk sample. Beyond the generic core cross- β structure (Dobson, 1999), it is not clear how the polypeptide subunits are arranged in the fibrils, or how the protofilaments interact. An alternative parallel structure, the β -helix, has been proposed to form amyloid fibrils (Wille *et al.*, 2002; Guo *et al.*, 2004), but direct experimental evidence for this structure is lacking.

4. EM Methods for Amyloid

4.1. Structural Information Depends on the Type of EM Sample Preparation

Examination of stained sections cut from tissue or bulk samples provides some information on the dimensions and protofilament substructures of amyloid fibrils. However, the structural features may be very distorted by this type of sample preparation. The two methods for preparing macromolecular assemblies for EM at molecular resolution are negative stain and cryo EM. In negative-stain EM, the sample is dried onto the support film in a solution of heavy metal stain. The stain provides good contrast of the outer surface of the structure, but the structure may be distorted by staining and dehydration. In cryo EM, the specimen is preserved in the native, hydrated state by flash freezing of a thin layer of solution, which is then imaged at low temperature. In negative stain EM, the sample is dried onto the support film in a solution of heavy metal stain. The method is quick and simple, and stain provides good contrast of the outer surface of the structure, but the structure may be distorted by staining and dehydration. In cryo EM, the specimen is preserved in the native, hydrated state by flash freezing of a thin layer of solution, which is then imaged at low temperature. Cryo EM does not necessarily give higher resolution, but provides more reliable structural information. It slows the rate of electron beam damage, the ultimate limitation on imaging of biological macromolecules,

and can reveal internal features of the density, such as α -helices and beta-sheets. In practice, the resolution is limited by the degree of disorder in the sample.

4.2. 3D Reconstruction from Electron Micrographs

The 3D structure of assemblies with helical symmetry can be determined from EM images (DeRosier and Klug, 1968; Stewart, 1988). If the assembly is well ordered, such as in bacterial flagella, close to atomic resolution can be achieved (Yonekura *et al.*, 2003). The basis for 3D reconstruction lies in the nature of the EM image and in the helical arrangement of subunits. The transmission EM image is a two-dimensional (2D) projection of the 3D structure (Figure 12.2-3a and b). An object can be reconstructed from its projections, if a series of projections at different viewing angles are available. In the case of a helix, the subunits forming the fibril are rotated through different angles around the fibril axis (Figure 12.2-3c), thus providing the range of view orientations of the repeating unit needed for 3D reconstruction by computed tomography. In theory, a single view of a long helical fibril is sufficient for 3D structure determination. In practice, unlike the image of a patient in medical computed tomography, images of different fibrils are averaged together, because the individual, raw images have a low signal to noise ratio. This arises from the extreme sensitivity of biological macromolecules to electron beam damage. Only very weak exposures can be taken of any given area. Weak exposures, combined with the very low contrast between unstained protein and ice, results in very noisy, low contrast images. Averaging over many similar projections is therefore

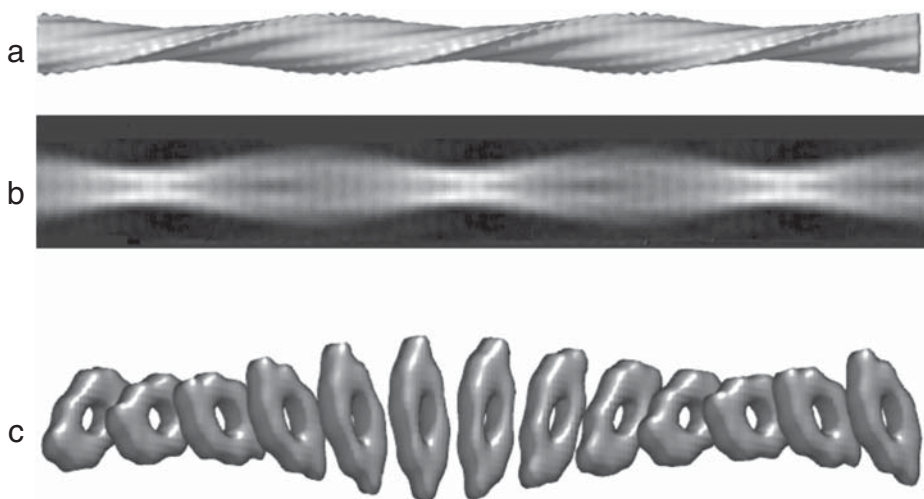


Figure 12.2-3. Surface and projection views of the cryo EM structure of an amyloid fibril (Jiménez *et al.*, 1999). (a) A surface-rendered view of an SH3 domain fibril, showing the 700-Å long twist. (b) 2D projection of the map in (a). The EM image is a 2D projection of the 3D structure. Protein density is light, and the dark central line indicates that there is a hollow core to the fibril. (c) The 3D map is chopped up into separate slices, to show the changing orientation of the structure along the helical axis. Each region contributes a different projection of the 3D structure to the image, thus providing the information needed to reconstruct the 3D density.

needed to obtain reliable information. A brief review of these methods can be found in Saibil (2000).

4.3. Sorting Out Structural Variations by Image Analysis

An advantage of imaging methods is that different fibril morphologies can be recognized and processed separately. But even in cases where the fibrils appear similar, the twist varies considerably along the length of a typical fibril (Jiménez *et al.*, 1999, 2001, 2002), so that established methods of helical reconstruction are ineffective. A way of sorting out this unwanted variation is to computationally chop up the fibril images into short segments with recognizable structural features. The segments can then be treated as independent objects ("single particles") and individually aligned and classified into similar subsets by multivariate statistical analysis, using the methods of single particle analysis (van Heel *et al.*, 2000; Frank, 2002) (Figure 12.2-4). Once homogeneous classes are obtained, helical symmetry can be applied to the average of all images within a class for 3D reconstruction. Jiménez *et al.* (1999) have developed this approach for amyloid fibrils, but there are also other, similar

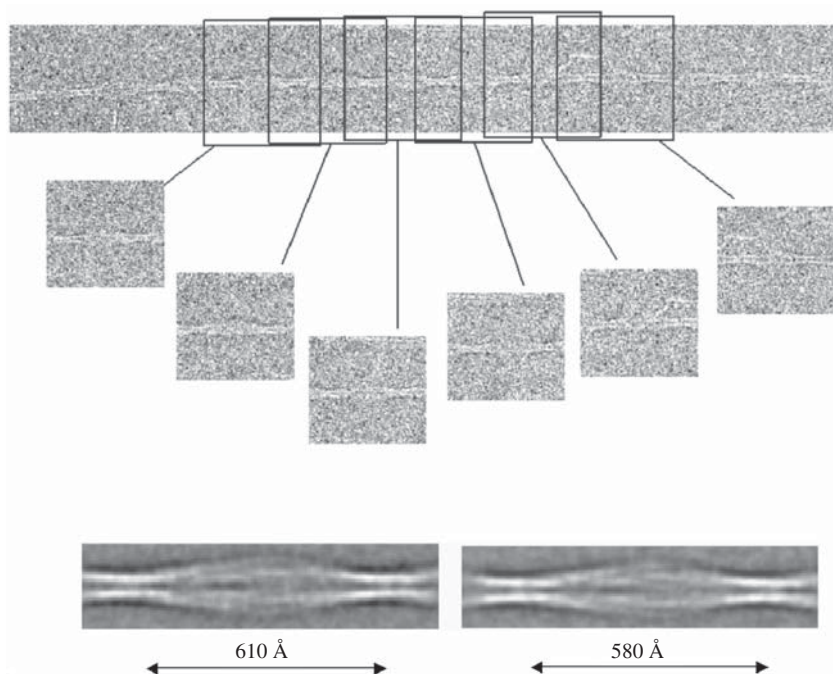


Figure 12.2-4. Single particle processing of a fibril. The red boxes show fibril segments being extracted from the cryo EM image of an SH3 fibril. When these are aligned, classified and averaged within subclasses, much more detail is revealed in the averaged views. Two class averages are shown, with a 30-Å difference in helical repeat. If all the segments are averaged together without classification, the different repeat lengths cause the structural details to be smeared out. Figure reproduced from Jiménez *et al.* (1999) by permission from Nature Publishing Group.

approaches for dealing with disordered helical structures (Crowther and Wischik, 1985; Bluemke *et al.*, 1988; Egelman, 2000).

5. Results of EM Studies

5.1. Protofilament Arrangement and Fibril Morphology

A range of two to six protofilaments has been reported from EM sectioning, fiber diffraction models, and cryo EM 3D reconstructions of various naturally occurring and model amyloid fibrils. EM sectioning has been used to study the arrangement of the laterally associated protofilaments (Cohen *et al.*, 1982; Kirschner *et al.*, 1987; Fraser *et al.*, 1991). A survey of protofilament arrangement in a wide range of fibrils suggested that they contained five or six protofilaments ~ 30 Å in diameter (Serpell *et al.*, 2000).

The first 3D reconstruction of an amyloid fibril was done by cryo EM of a model fibril grown from an SH3 (Src homology 3) domain (**Figure 12.2-5; see color insert**) (Jiménez *et al.*, 1999). Although the preparation contained many different fibril morphologies, an abundant form with a prominent twist appeared to be the most ordered. The structure was determined to about 25-Å resolution by a combination of single-particle analysis and helical reconstruction as described in the previous section. The fibril is formed by four protofilaments, each with a 20×40 -Å cross-section, wound around a hollow core 50 Å in diameter at its widest. Two independent reconstructions, from 580 and 610-Å repeats, are very similar. The native SH3 fold is a β -sandwich, but the overall shape is globular and too wide to fit into the protofilament density. It must be unfolded and adopt a more extended shape to fit into the high density regions of the fibril. A model for the polypeptide fold in the fibril was suggested, in which β -sheets from the β -sandwich fold of the native structure were reoriented and the β -strands were straightened. In the model, the β -sheets contain a mixture of parallel and antiparallel strands. They fit well into the protofilament density and the loops provide the right amount of mass to account for the remaining density. The hand of the fibril twist was not determined in this study. On the basis of subsequent results, it is likely that the SH3 fibrils are left-handed like other amyloid fibrils (see next section), and not right-handed, as shown in the paper by Jiménez *et al.* (1999).

In another study, 3D reconstructions of amyloid were generated from cryo EM images of insulin fibrils (Jiménez *et al.*, 2002). Diverse morphologies with two to six protofilaments were found in a single sample, but the protofilament shape appeared similar in all the structures. Only one of these morphologies was present in sufficient abundance to allow averaging of repeats from many fibrils (**Figure 12.2-6; see color insert**). In this case, four protofilaments are wound together in a compact arrangement. The presence of two interchain and one intrachain disulfide bonds places strong constraints on the topology when the almost completely α -helical molecule refolds into a β -conformation in the fibrils. The disulfide-linked strands must run in parallel, and the intrachain disulfide must impose some curvature on one of the sheets. With these constraints, a schematic model was proposed for the backbone conformation in these fibrils.

Recently, 3D structures of two more *in vitro* grown amyloid fibrils have been determined by the authors and their colleagues. Two intertwined protofilaments with connecting bridges of density were seen in a 3D reconstruction of negatively stained amyloid fibrils formed from recombinant mammalian prion protein (Tattum, Cohen-Krausz, Khalili-Shirazi, Orlova, Jackson, Clarke, Collinge, Saibil, unpublished). A more complex, extended structure with six protofilaments has been determined from cryo EM images of $\beta 2$ -microglobulin amyloid fibrils (Cohen-Krausz, Hodgkinson,

Orlova, McParland, Jahn, Radford, Saibil, unpublished). Notably, these new structures show evidence of a subunit repeat along the fibrils.

With the accumulation of fibril data, it is clear that amyloid fibrils can be composed of different numbers of laterally associated protofilaments. The growth conditions can influence their morphology, but even when prepared under identical conditions, the resulting fibrils may show a range of structures. Although it was previously thought that *ex vivo* amyloid fibrils might be more uniform in structure than those assembled *in vitro*, a cryo EM study of mutant apolipoprotein A1 and lysozyme fibrils showed similar variability (Jiménez *et al.*, 2001).

5.1.1. The β -Strands in Amyloid Fibrils Stack with a Slight, Left-Handed Twist

Because EM images are projections through the structure, individual images do not reveal the handedness. The hand can be determined by recording pairs of images at different tilt angles, by metal shadowing EM, or by atomic force microscopy to image only the top surface of the fibril. In all cases where this has been done, the fibril twist has been found to be left-handed. This is important, because the twist of the protofilaments must be related to the underlying twist of the β -sheet, to maintain the contacts between protofilaments in the fibril assembly (**Figure 12.2-7A and B; see color insert**). Contacts between protofilaments are practically a universal feature of amyloid fibrils, and single protofilaments are rarely seen. To maintain the interprotofilament contacts along the length of the ribbon-like or twisted fibrils, the β -sheet twist must follow the long-range twist of the protofilaments. The common, naturally occurring configuration of β -sheets has a left-handed twist. This is seen by the counterclockwise twist of the sheet when viewed edge-on (**Figure 12.2-7C; see color insert**). The strands also stack with a left-handed twist in the perpendicular view in Figure 12.2-7D. Because the direction of twist depends on the direction of view, it is sometimes stated that β -sheets are right-handed. In Figure 12.2-7E, the sheet is turned by 90° , in the plane of the page, from the view in Figure 12.2-7D. In this view, the sheet has a right-handed twist. A further implication of the twist relationship is that the β -sheets are nearly flat in the fibrils—with a typical distance of $\sim 1000 \text{ \AA}$ for a 180° turn (distance between crossovers), the angle between successive β -strands is on the order of 1° .

5.2. Prion Amyloid Models from Crystalline Arrays

Although prion fibrils or rods appear to be the most abundant form in natural deposits, 2D arrays have been found in infectious, *ex vivo* specimens purified from scrapie brain tissue (Wille *et al.*, 2002). A 2D negative-stain EM analysis revealed a hexagonal lattice of circular structures. On the basis of differential staining, sugar labeling, and comparison of samples made from different length constructs of a prion protein (PrP), a detailed β -helix model has been proposed for the structure of PrP^{Sc} in these arrays (Govaerts *et al.*, 2004).

3D nanocrystals of a yeast prion peptide provide a different experimental model. The peptide GNNQQNY from the N-terminal region of the yeast prion protein Sup35, which forms amyloid fibrils, colloidal aggregates, and highly ordered nanocrystals has been studied as a model system for characterizing the cross- β conformation (Diaz-Avalos *et al.*, 2003). High-resolution electron diffraction patterns from single nanocrystals and X-ray powder diffraction patterns are consistent with a tightly packed cross- β arrangement with low hydration.

5.3. The Structure of Intermediate Oligomers—The Toxic Agent?

Globular assemblies around 100 Å in diameter are typically seen in association with fibril growth *in vitro*, and these smaller complexes rather than mature amyloid fibrils are thought to be the cytotoxic agent in amyloid disease (Bucciantini *et al.*, 2002; Lashuel *et al.*, 2002; Kayed *et al.*, 2003, 2004). They could represent an intermediate state on the pathway to fibril formation or an alternative final product of aggregation. Short, linear aggregates of these blobs appear to form immature fibrils (protofibrils) (Harper *et al.*, 1999). They are variable in size and shape, and in some cases ring structures are seen (Figure 12.2-8), for example, in preparations of α -synuclein and β -amyloid (A β) (Lashuel *et al.*, 2002, 2003). Spectroscopic analysis shows that these oligomers are rich in β -structure (Lashuel *et al.*, 2002). It has been suggested that the rings and other small oligomers form membrane pores and that membrane permeabilization is an important mechanism of neurotoxicity in amyloid disease (Lashuel *et al.*, 2002; Kaye *et al.*, 2004). A striking finding is that specific antibodies recognize small oligomers and protofibrils from a range of amyloid precursor proteins, but not the mature fibrils formed by the same oligomers (Kayed *et al.*, 2003). Not only do the fibrils themselves have

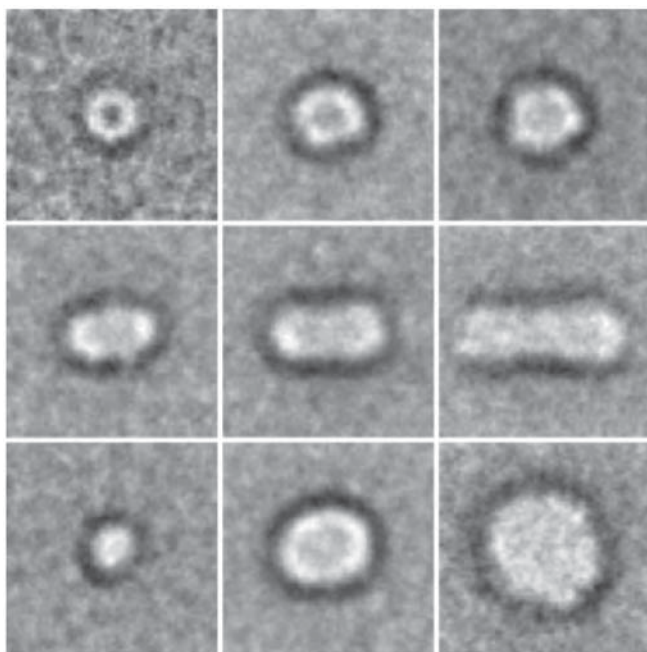


Figure 12.2-8. Negative-stain EM images of early oligomers and protofibrils (Lashuel *et al.*, 2003). A set of averaged views indicates the variety of oligomeric structures found, including ring-shaped structures that may act as membrane pores. The size of each box is 400 Å. Image provided by Thomas Walz and reproduced by permission of Elsevier Press.

generic structural features (Dobson, 1999) and antigenic properties (O'Nuallain and Wetzel, 2002), but the toxic fibril precursors have common antigenic properties. The oligomer antibody specifically abolishes toxicity in a cell assay (Kayed *et al.*, 2003).

6. Prospects for Fibril Structure Determination

Electron microscopy is an important tool in characterizing amyloid fibril morphology and the only method that has so far provided 3D structures of fibrils, albeit at low resolution. Negatively stained specimens are readily prepared and reveal the type of aggregates present in the sample, the degree of fibril formation and their morphology. Cryo EM, although more complicated to implement, can provide high-resolution information on the native, hydrated specimen. Unlike other structural methods, EM can deal with fibril heterogeneity and disorder, although these problems provide the main limitation on resolution. If growth conditions can be found that produce highly ordered fibrils, then high resolution 3D structures could be obtained. Finally, atomic structures of fibril subunits determined by other methods can be incorporated by docking them into EM maps to build up a structural model of the assembly.

7. Abbreviations

2D	Two-dimensional
3D	Three-dimensional
EM	Electron microscopy
PrP	Prion protein

References

- Bluemke, D.A., Carragher, B., and Josephs, R. (1988). The reconstruction of helical particles with variable pitch. *Ultra-microscopy* 26:255–270.
- Bucciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C.M., and Stefani, M. (2002). Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature* 416:507–511.
- Crowther, R.A., and Wischik, C. 1985. Image reconstruction of the Alzheimer paired helical filament. *EMBO J.* 4:3661–3665.
- Cohen, A.S., Shirahama, T., and Skinner, M. (1982). Electron microscopy of amyloid. In: Harris, J.R. (Ed.), *Electron microscopy of proteins*, vol. 3. London: Academic Press, pp. 165–205.
- DeRosier, D.J., and Klug, A. (1968). Reconstruction of three dimensional structures from electron micrographs. *Nature* 217:130–134.
- Diaz-Avalos, R., Long, C., Fontano, E., Balbirnie, M., Grothe, R., Eisenberg, D., and Caspar, D.L.D. (2003). Cross-beta order and diversity in nanocrystals of an amyloid-forming peptide. *J. Mol. Biol.* 330:1165–1175.
- Dobson, C.M. (1999). Protein misfolding, evolution and disease. *Trends Biochem. Sci.* 24:329–332.
- Egelman, E.H. (2000). A robust algorithm for the reconstruction of helical filaments using single-particle methods. *Ultramicroscopy* 85:225–234.

- Frank, J. (2002). Single-particle imaging of macromolecules by cryo-electron microscopy. *Annu. Rev. Biophys. Biomol. Struct.* 31:303–319.
- Fraser, P.E., Nguyen, J.T., Surewicz, W.K., and Kirschner, D.A. (1991). pH-dependent structural transitions of Alzheimer's amyloid peptides. *Biophys. J.* 60:1190–1201.
- Goldsbury, C.S., Cooper, G.J.S., Goldie, K.N., Müller, S.A., Saafi, E.L., Gruijters, W.T.M., Misur, M.P., Engel, A., and Aebi, U. (1997). Polymorphic fibrillar assembly of human amylin. *J. Struct. Biol.* 119:17–27.
- Govaerts, C., Wille, H., Prusiner, S.B., and Cohen, F.E. (2004). Evidence for assembly of prions with left-handed β -helices into trimers. *Proc. Natl. Acad. Sci. USA* 101:8342–8347.
- Guo, J.T., Wetzel, R., and Xu, Y. (2004). Molecular modeling of the core of Abeta amyloid fibrils. *Proteins* 57: 357–364.
- Harper, J.D., Wong, S.S., Lieber, C.M., and Lansbury, P.T., Jr. (1999). Assembly of A beta amyloid protofibrils: an in vitro model for a possible early event in Alzheimer's disease. *Biochemistry* 38:8972–8980.
- Jaroniec, C.P., MacPhee, C.E., Bajaj, V.S., McMahon, M.T., Dobson, C.M., and Griffin, R.G. (2004). High resolution molecular structure of a peptide in an amyloid fibril determined by magic angle spinning NMR spectroscopy. *Proc. Natl. Acad. Sci. USA* 101:711–716.
- Jiménez, J.L., Guijarro, J.I., Orlova, E., Zurdo, J., Dobson, C.M., Sunde, M., and Saibil, H.R. (1999). Cryo-electron microscopy structure of an SH3 amyloid fibril and model of the molecular packing. *EMBO J.* 18:815–821.
- Jiménez, J.L., Tennent, G., Pepys, M., and Saibil, H.R. (2001). Structural diversity of *ex vivo* amyloid fibrils studied by cryo-electron microscopy. *J. Mol. Biol.* 311:241–247.
- Jiménez, J.L., Nettleton, E.J., Bouchard, M., Robinson, C.V., Dobson, C.M., and Saibil, H.R. (2002). The protofilament structure of insulin amyloid fibrils. *Proc. Natl. Acad. Sci. USA* 99:9196–9201.
- Kayed, R., Head, E., Thompson, J.L., McIntire, T.M., Milton, S.C., Cotman, C.W., and Glabe, C.G. (2003). Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science* 301:1847–1849.
- Kayed, R., Sokolov, Y., Edmonds, B., McIntire, T.M., Milton, S.C., Hall, J.E., and Glabe, C.G. (2004). Permeabilization of lipid bilayers is a common conformation-dependent activity of soluble amyloid oligomers in protein misfolding diseases. *J. Biol. Chem.* 279:46363–46366.
- Kirschner, D.A., Inouye, H., Duffy, L.K., Sinclair, A., Lind, M., and Selkoe, D.J. (1987). Synthetic peptide homologous to β -protein from Alzheimer's disease forms amyloid like fibrils in vitro. *Proc. Natl. Acad. Sci. USA* 84:6953–6957.
- Lashuel, H.A., Petre, B.M., Wall, J., Simon, M., Nowak, R.J., Walz, T., and Lansbury, P.T., Jr. (2002). α -Synuclein, especially the Parkinson's disease-associated mutants, forms pore-like annular and tubular protofibrils. *J. Mol. Biol.* 322:1089–1102.
- Lashuel, H.A., Hartley, D.M., Petre, B.M., Wall, J.S., Simon, M.N., Walz, T., and Lansbury, P.T., Jr. (2003). Mixtures of wild-type and a pathogenic (E22G) form of A β 40 in vitro accumulate protofibrils, including amyloid pores. *J. Mol. Biol.* 332:795–808.
- O'Nuallain, B., and Wetzel, R. (2002). Conformational Abs recognizing a generic amyloid fibril epitope. *Proc. Natl. Acad. Sci. USA* 99:1485–1490.
- Saibil, H.R. (2000). Macromolecular structure determination by cryo-electron microscopy. *Acta Crystallogr. D* 56:1215–1222.
- Serpell, L.C., and Smith, J.M. (2000). Direct visualization of the β -sheet structure of synthetic Alzheimer's amyloid. *J. Mol. Biol.* 299:225–231.
- Serpell, L.C., Sunde, M., Benson, M.D., Tennent, G.A., Pepys, M.B., and Fraser, P.E. (2000). The protofilament substructure of amyloid fibrils. *J. Mol. Biol.* 300:1033–1039.
- Shirahama, T., and Cohen, A.S. (1967). High resolution electron microscopic analysis of the amyloid fibril. *J. Cell Biol.* 33:679–706.
- Stewart, M. (1988). Computer processing of electron micrographs of biological structures with helical symmetry. *J. Electron Microsc. Tech.* 9:325–358.
- Sunde, M., Serpell, L.C., Bartlam, M., Fraser, P.E., Pepys, M.B., and Blake, C.C.F. (1997). The common core structure of amyloid fibrils by synchrotron X-ray diffraction. *J. Mol. Biol.* 273:729–739.
- Tycko, R. (2004). Progress towards a molecular-level structural understanding of amyloid fibrils. *Curr. Opin. Struct. Biol.* 14:96–103.
- van Heel, M., Gowen, B., Matadeen, R., Orlova, E.V., Finn, R., Pape, T., Cohen, D., Stark, H., Schmidt, R., Schatz, M., and Patwardhan, A. (2000). Single-particle electron cryo-microscopy: towards atomic resolution. *Q. Rev. Biophys.* 33:307–369.

- Wille, H., Michelitsch, M.D., Guénebaut, V., Supattapone, S., Serban, A., Cohen, F.E., Agard, D.A., and Prusiner S.B. (2002). Structural studies of the scrapie prion protein by electron crystallography. *Proc. Natl. Acad. Sci. USA* 99:3563–3568.
- Yonekura, K., Maki-Yonekura, S., and Namba, K. (2003). Complete atomic model of the bacterial flagellar filament by electron cryomicroscopy. *Nature* 424:643–650.

Atomic Force Microscopy

Justin Legleiter and Tomasz Kowalewski

1. Abstract

The ultimate objective of the amyloid fibril studies is to elucidate the physicochemical aspects and molecular mechanisms of pathological self-assembly of biological macromolecules. *In situ* atomic force microscopy (AFM) is a useful tool in studying the aggregation of peptides associated with various conformational diseases under a wide variety of conditions. The unique capability of *in situ* AFM is the direct visualization of the behavior of biological macromolecules at solid–liquid interfaces, under nearly physiological conditions. AFM can provide information that is not easily accessible by other methods due to the unique ability to follow in time three-dimensional nanoscale surface maps. It is of particular importance for the analysis of the roles of surfaces (including supported lipid bilayers and cells) in the processes leading to pathological peptide assembly and the structural consequences of the addition of modulating factors on such aggregation. Used in conjunction with other techniques, AFM has become an invaluable tool, providing useful information about disease-related protein aggregation.

2. Introduction

There are a large and diverse number of diseases that are commonly classified as conformational diseases. The common feature of these diseases is the rearrangement of a specific protein to a nonnative conformation that promotes aggregation and deposition within tissues or cellular compartments. Such diseases include Alzheimer's (AD) and Parkinson's diseases (PD), amyloidoses, α 1-antitrypsin deficiency, the prion encephalopathies, and many more (Crowther, 2002; Žerovnik, 2002). A common structural motif in the majority of these diseases is the emergence of extended, β -sheet-rich, proteinaceous fibrillar aggregates that are commonly referred to as amyloids (Sunde and Blake, 1998; Žerovnik, 2002). These fibrillar species are made up of intertwined protofibrillar filaments, which often have globular, soluble protein aggregate precursors (Crowther, 2002; Žerovnik, 2002). The nature and location of aggregates in diseased tissue is dependent on the particular protein involved, and there are often genetic mutations or problematic processing that can be correlated to these aggregation events. For the vast majority of these diseases, there are no widely effective preventative measures or therapeutic treatments. Due to the progression of protein aggregates from globular precursors to mature fibrils, there is an active debate as to which type of aggregate is the most toxically relevant form of the misfolded peptide. Hence, a technique that would allow for distinguishing different morphological features of these aggregates and simultaneously investigating other properties and interactions would be of enormous benefit in studying conformational diseases. In recent years, atomic force microscopy (AFM) has emerged as a technique that could be particularly useful in addressing these issues.

In the past decade, AFM has become an increasingly used technique in the study of conformational diseases, both as an auxiliary and primary tool. It has provided particularly useful insights due to its unique ability to be operated not only in air (*ex situ*) but also in solution (*in situ*), making it possible to directly visualize the behavior of biological macromolecules at solid–liquid interfaces, under nearly physiological conditions. This review describes recent progress in the use of AFM in the area of conformational diseases, with particular focus on two systems where it was applied most extensively: β -amyloid ($A\beta$) and α -synuclein (α -syn), proteins respectively implicated in AD and PD.

3. AFM

Since its invention in 1986 (Binnig *et al.*, 1986), AFM has become an increasingly more important technique in exploring biological phenomena at the nanoscale. Principal schemes and components of AFM are shown in Figure 12.3-1. The laser beam produced by the laser diode is reflected off the back of a cantilever and then focused onto the position-sensitive photodetector, acting as an optical lever that can be used to determine the vertical displacement of the cantilever with subangstrom resolution. Three major modes of operation for AFM are: (a) contact, (b) tapping (or intermittent contact), and (c) noncontact. Owing to its ability to reduce lateral forces, the tapping mode is the most commonly used in biological studies, and will be described here. The cantilever is oscillated near its resonance frequency ω_0 , and the tip is allowed to intermittently contact the surface, leading to the decrease of cantilever oscillation amplitude from the “free” amplitude A_0 to some “tapping” amplitude A . An image is acquired by raster scanning the tip over the sample while adjusting the vertical displacement of the scanner to maintain the constant value of set-point ratio $s = A/A_0$. The feedback loop allows for the precise control of the force between the tip and sample, allowing for minimization of the interaction force and providing the ability to perform force-dependent experiments. Intermittent contact AFM can be easily implemented under liquids (Hansma *et al.*, 1994; Putman *et al.*, 1994; Lantz *et al.*, 1999). Due to the resulting unique ability to operate under nearly

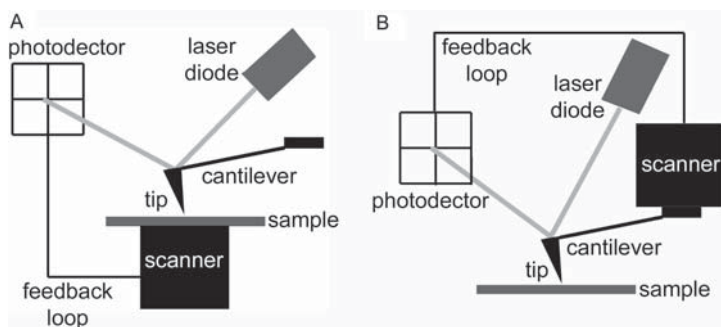


Figure 12.3-1. The two most common configurations of an AFM: (A) scanned sample and (B) scanned cantilever. In configuration (B), the scanner/cantilever assembly can be easily positioned over different areas of large samples such as whole wafers or Petri dishes. In addition, it can be integrated with inverted optical microscopes. These features make it particularly attractive for biological imaging

physiological conditions, AFM is particularly suitable to study the morphology of biological samples and follow its evolution over time (Goldsbury *et al.*, 1999; Malkin *et al.*, 1996). In addition, AFM does not require extensive sample preparations (i.e., staining, gold sputtering, freezing, etc.) that are commonly used with other forms of microscopy, which are all potential sources of artifacts and perturbations.

4. Studies of A β Peptide Aggregation and Morphology

The ordered aggregation of the A β peptide in the brain as plaques consisting of fibrils is a hallmark of AD, a late-onset neurodegenerative disease (Selkoe, 2001). A β is a cleavage product of the amyloid precursor protein (APP), a transmembrane protein containing 677–770 amino acids. The insoluble aggregated form of A β , which deposits in the extracellular space in the brain and on the walls of cerebral blood vessels (Lansbury, 1996), exhibits an enhanced β -sheet content as opposed to the partially α -helical soluble form found in body fluids (Kelly, 1998; Smith, 1998). The A β component of amyloid plaques found in the diseased brain consist primarily of two versions of the peptide, which are 40 and 42 amino acids long and are referred to as A β (1–40) and A β (1–42). Of the two, A β (1–42) tends to aggregate more quickly than A β (1–40) (Jarrett *et al.*, 1993). Despite the lack of the definitive proof of the causative role of A β in AD, it is generally agreed that its aggregation and deposition play a central role in this disease. In recent years, AFM has been playing an increasingly important role in studies of the physicochemical aspects of A β aggregation.

4.1. A β Aggregation and Fibrillization

4.1.1. Commonly Observed A β Morphologies

There are several common structures and morphologies of A β at different stages of aggregation that have been observed by *ex situ* AFM. Small, prefibrillar, globular aggregates have often been observed for freshly incubated A β solutions [both A β (1–40) and A β (1–42)] (Roher *et al.*, 1996; Harper *et al.*, 1997a, 1997b; Blackley *et al.*, 1999; Nichols *et al.*, 2002). These globular aggregates were observed to give way to prefibrillar or protofibrillar aggregates with diameters of ~3–5 nm; these aggregates were, in turn, observed to decrease in number at longer incubation times as mature fibrils developed (Harper *et al.*, 1997b; Blackley *et al.*, 1999). AFM observations revealed that the thickness and height of mature fibrils range respectively from 7–12 nm and from 4–9 nm (Stine *et al.*, 1996). Their lengths reached up to several or even tens of microns. These mature fibrils often displayed distinct longitudinal periodicities (usually ~25 or ~43 nm), with occasional splitting defects indicating twisted structure consisting of two or more protofibrils (Roher *et al.*, 1996; Stine *et al.*, 1996; Harper *et al.*, 1997a, 1997b). Such twisted structure was further supported by the appearance of branching along mature fibrils (Stine *et al.*, 1996; Harper *et al.*, 1997a, 1997b; Blackley *et al.*, 1999).

In situ AFM observations in physiological buffer solutions provide the unique opportunity to observe the development of aggregates over time. Small, highly mobile, globular aggregates of both A β (1–40) (Blackley *et al.*, 2000) and A β (1–42) (Kowalewski and Holtzman, 1999) were observed on mica. With time, they organized into elongated prefibrillar aggregates that continued to grow in length (Figure 12.3-2). Aggregates of A β (1–42) forming on graphite in solutions were markedly different in their morphology than those observed on mica (Figure 12.3-3) (Kowalewski and Holtzman, 1999). A β (1–42) formed extended nanoribbons with heights of 1–1.2 nm and widths of ~18 nm, which

suggested that peptide chains adopted fully extended β -sheet conformation and were perpendicular to the long axis of nanoribbons. These putative β -sheets elongated over time and preferentially organized into parallel “rafts” while maintaining preferential alignment along three equivalent directions, most likely pointing to a templating effect of the graphite lattice. Described *ex situ* and *in situ* AFM experiments suggest a pathway of A β aggregation that begins with oligomers that can further aggregate into prefibrillar filaments or protofibrils, which in turn, further aggregate and intertwine to form mature fibrils.

4.1.2. Seeding and Amyloidogenic Peptide Fragments

Atomic force microscopy has been used to study the formation of different types of aggregates of A β in a time-dependent manner under various conditions. It was shown that A β (1–40) formed fibrils at a slower rate than A β (1–42) (Harper *et al.*, 1997b), and that preformed fibrils, but not protofibrils, could seed fibril growth in A β solutions (Harper *et al.*, 1997a). Atomic force microscopy and thioflavin T (ThT) fluorescence experiments involving coaggregation of A β (1–40) with different A β fragments pointed to the critical role of residues 17–20 and 30–35 (Liu *et al.*, 2004). In particular, the A β (25–35) fragment was observed to increase the formation of oligomers and protofibrils, and to aggregate by itself to form long, thin filaments.

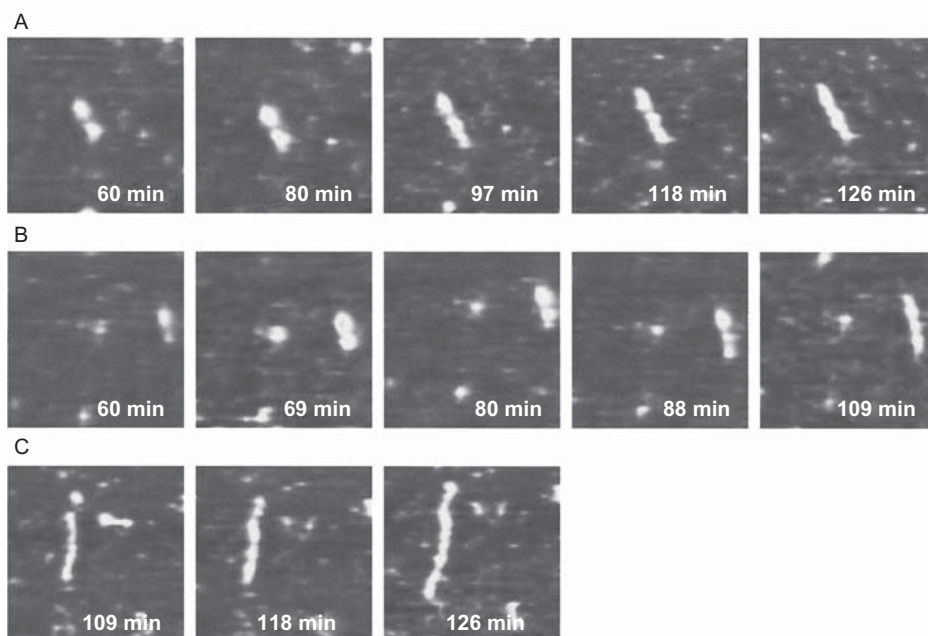


Figure 12.3-2. *In situ* tapping mode AFM makes it possible to track the early steps of A β aggregation. The above $1\text{-}\mu\text{m} \times 1\text{-}\mu\text{m}$ images show how compact, globular A β aggregates organize into elongated forms. Series A–C show the evolution of three different aggregates. (From Blackley *et al.*, 2000. Copyright 2000, with permission from Elsevier.)

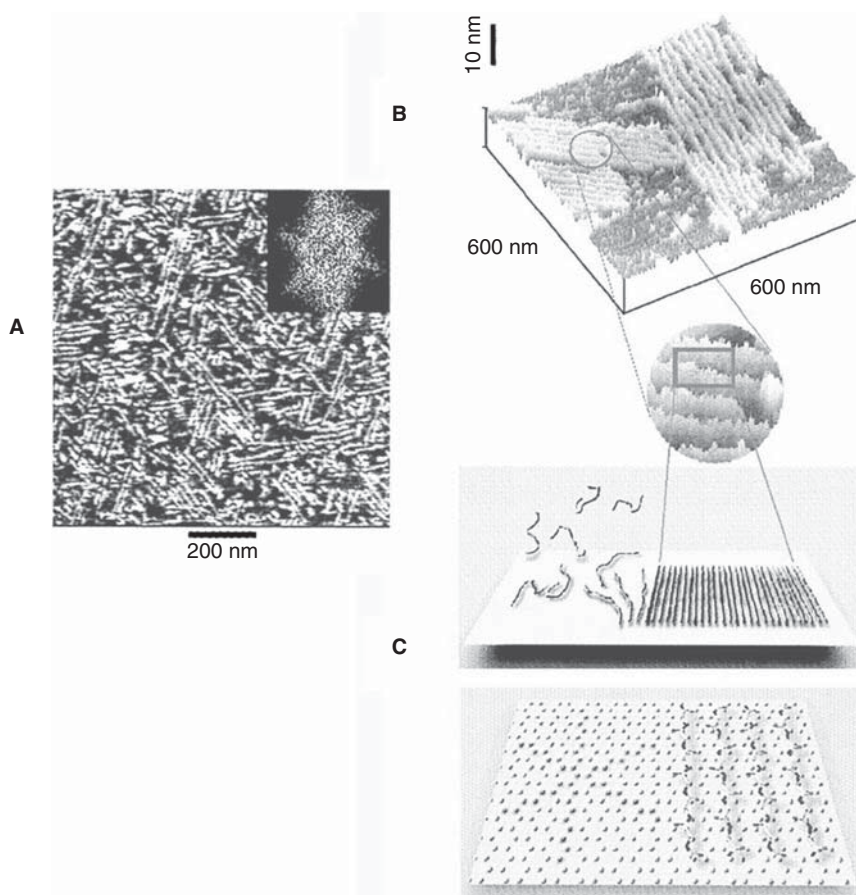


Figure 12.3-3. *In situ* AFM images of aggregates of A β (1–42) formed in PBS buffer solutions in contact with highly oriented pyrolytic graphite. (A) Dense layer of elongated nanoribbons preferentially oriented along three directions. The threefold symmetry of this arrangement corresponds to the crystallographic symmetry of the graphite surface (C). Based on the 2D Fourier transform analysis (inset), the lateral spacing between the long axis of nanoribbons is ~ 18 nm. (B) 3D rendering of aggregates of nanoribbons partially covering graphite surface. The height of individual nanoribbons is 1.0–1.2 nm. The dimensions of such aggregates on graphite strongly suggest that A β adopts a β -sheet form with peptide chains perpendicular to the long axis of the ribbon. (From Kowalewski and Holtzman, 1999.)

4.2. Factors Modulating A β Aggregation

4.2.1. Role of Solution Conditions

A comprehensive AFM investigation of the influence of various solution and incubation conditions (time, concentration, temperature, pH, ionic strength, and A β species) on the aggregation of A β was reported by Stine *et al.* (2003). Two aggregation protocols were used: one resulting in fibrils, and

another leading to oligomers or amyloid-derived diffusible ligands (ADDLs), which may be of the same class as the globular aggregates mentioned previously. For fibril formation, acidic pH conditions were used. To form ADDLs, A β was incubated in cell culture medium (phenol red-free Ham's F-12). In both cases, the incubation time necessary for the appearance of A β (1–42) aggregates decreased with an increase in A β concentration (Figure 12.3–4). Incubation temperature was also found to influence A β (1–42) assembly. Under oligomer-forming conditions, uniform oligomers with heights of ~2–4 nm were observed following a 24-hour incubation at 4°C; at room temperature, a small number of protofibrils was observed. Increase of incubation temperature to 37°C led to the increase of the number of protofibrils, but no extended fibrils were ever observed. For fibril-forming conditions, incubations at 37°C for 24 hours resulted in a uniform population of 4 nm tall, extended fibrils. At lower temperatures, the number of fibrils decreased significantly. The assembly process was also shown to be dependent on ionic strength and pH. A β (1–42) was incubated at 37°C at combinations of acidic and neutral pH with both low and physiological ionic strength. Extended fibrils formed at high pH and low ionic strength. Dense fibrillar aggregates were present in samples incubated at high pH with physiological ionic strength. At neutral pH and low ionic strength, A β remained mostly oligomeric with scattered fibrils present; however, when the ionic strength was high, the oligomers appeared to coalesce into small aggregates with a height of ~5–8 nm and some protofibrils.

4.2.2. Interactions of A β with Lipids

Particularly useful insights were obtained in AFM studies of the effect of A β on model membranes and cells. One of the proposed mechanism for A β toxicity points to its ability to change cellular ion concentrations, calcium in particular, possibly through the formation of some kind of membrane channels. To explore this possibility, A β (1–42) (Rhee *et al.*, 1998) and A β (1–40) (Lin *et al.*, 1999) were incorporated into phospholipids vesicles that were imaged with *in situ* AFM. Atomic force microscopy probing of mechanical properties of these vesicles indicated that they stiffened in the presence of calcium, presumably due to such factors as: calcium ion-induced charge–charge repulsion inside vesicles, binding of calcium to lipids and proteins, and an enhanced efficiency of lipid–protein interactions. Interestingly, this increased stiffness was not observed when the vesicles were pretreated with anti-A β antibodies, Tris, or zinc, all of which are expected to block putative calcium channels. When A β (1–42) was reconstituted with a planar lipid bilayer, multimeric channel-like structures with symmetry suggesting tetramer or hexamer character were observed (Lin *et al.*, 2001). Studies conducted to determine the effect of A β (1–40), A β (1–42), and A β (25–35) on endothelial cells (Bhatia *et al.*, 2000) demonstrated that these cells underwent morphological changes in the presence of all three peptides with the highest sensitivity to A β (1–42). Cell disruption was observed at nanomolar concentration for A β (1–42). In contrast, micromolar concentrations of A β (1–40) were needed to trigger similar effects. Analogous observations were reported for fibroblasts in the presence of a nanomolar concentration of A β (1–42); morphological changes along the periphery of the cell were observed within 10–15 minutes (Bhatia *et al.*, 2000). Importantly, cell disruption could be prevented by anti-A β antibodies, zinc, and the removal of calcium.

Studies of the interaction of A β (1–40) with bilayers formed from total brain lipid extract (TBLE) revealed that, initially, it partially inserted into such bilayers and subsequently grew into small fibers without causing disruption (Yip and McLaurin, 2001). In addition, larger fiber-like structures associated with small bilayer disruptions were observed. With time, mature A β fibrils were observed, and in their presence, the bilayers underwent extensive disruption. The large fibrils were associated with the edges of the disrupted bilayer and were highly branched. The bilayers appeared to nucleate and enhance fibril growth, as fibrils were not detected in solution of A β (1–40) in their absence. Notably, preformed fibrils did not disrupt the bilayers, indicating that the actual self-

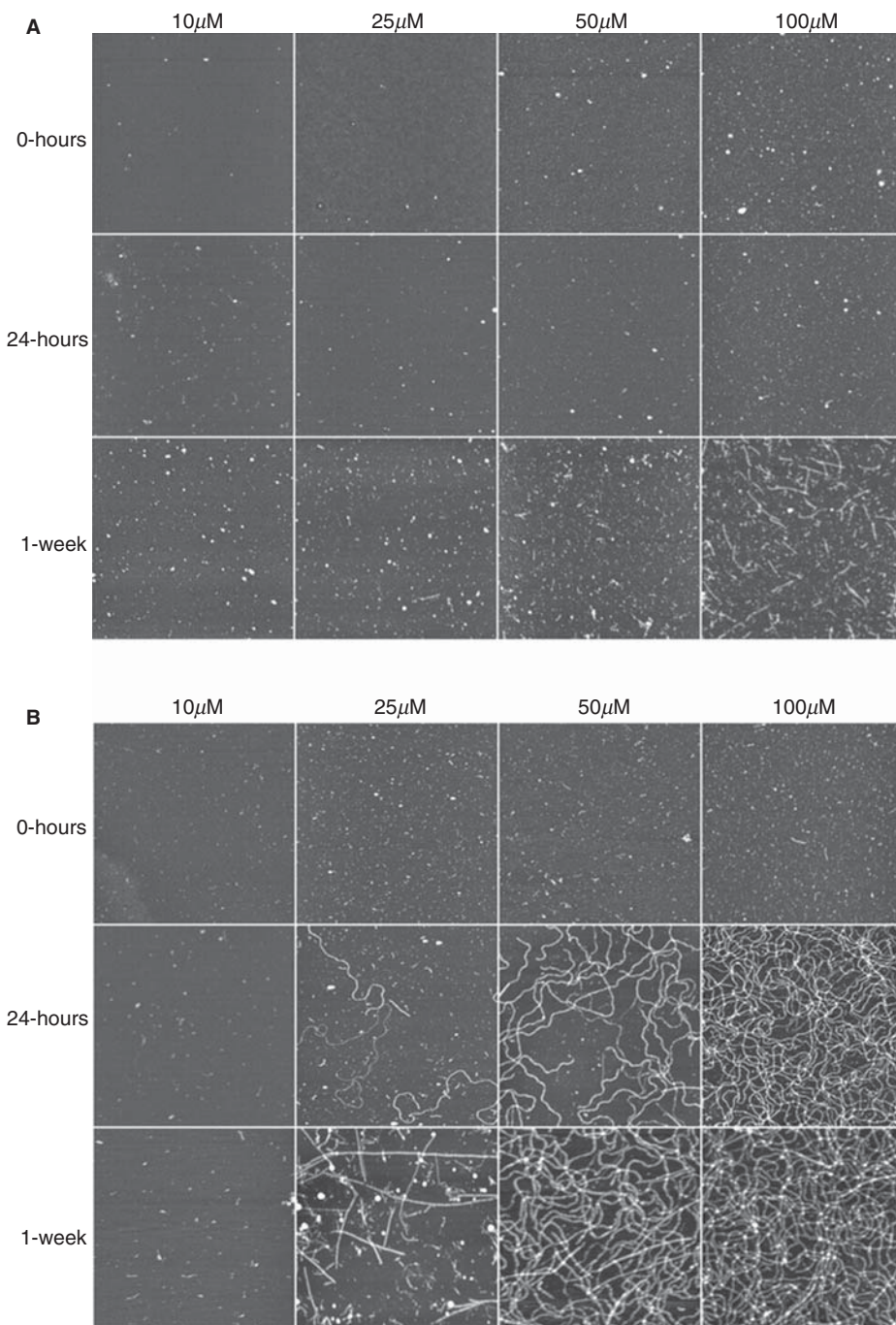


Figure 12.3-4. Dependence of A β (1–42) assembly on time and concentration for oligomer (cell culture medium, incubation at 4°C for 24 hours) and fibril forming (10 mM HCl, incubation at 37°C for 24 hours) conditions. Ten-, 25-, 50-, and 100- μ M solutions of A β (1–42) were incubated at (A) oligomer-forming conditions and (B) fibril-forming conditions. Solutions were monitored using AFM immediately after dilution, at 24 hours, and after 1 week. (From Stine *et al.* Journal of Biological Chemistry. Copyright 2003 by the American Society for Biochemistry & Molecular Biology. Reproduced with permission from the American Society for Biochemistry & Molecular Biology, in the form of Textbook via the Copyright Clearance Center.)

assembly process was key in membrane disruption. Control experiments with A β (1–40) were carried out with DMPC bilayers. Globular aggregates associated with small bilayer holes that underwent further aggregation were observed in this case. Fibril growth and/or extensive bilayer disruption was not observed here.

When compared to A β (1–40), A β (1–42) interacted differently with TBLE bilayers (Yip *et al.*, 2002). Thirty minutes after addition of A β (1–42) to the bilayers, small features proposed to be discrete molecules of A β (1–42) could be detected on the surface; after 7 hours, they were replaced by distinctly larger aggregates. Defects were rarely detected in the membrane in the presence of A β (1–42). When the amount of cholesterol in the bilayers was increased, more A β (1–42) deposited on the surface, but disruption was still not observed. When the bilayers were depleted of cholesterol, A β (1–42) aggregated in the interior of the bilayer, forming distinct domains revealed by changes in contrast in phase images.

Further studies of TBLE bilayers explored the effect of cholesterol (Yip *et al.*, 2001). Susceptibility of TBLE bilayers to A β (1–40)-induced disruption and their ability to induce its fibrillogenesis turned out to strongly depend on their cholesterol content. Neither the bilayers in which cholesterol content was increased by the addition of up to 30% exogenous cholesterol, nor cholesterol depleted bilayers, were disrupted by A β (1–40) over a time of 9–15 hours. When A β (1–40) was added to total brain lipid bilayers with 10% exogenous cholesterol, species proposed to correspond to discrete A β (1–40) peptides appeared on the bilayer after ~30 minutes. After 15 hours, ring-like A β (1–40) structures with diameters of 55–80 nm as well as short fibrils and small aggregates were observed. Interestingly, no membrane disruption was observed. At higher cholesterol content (30% of the total lipid), no large and ring-like aggregates of A β (1–40) aggregates were observed. When A β (1–40) was added to cholesterol depleted bilayers, fibrils grew but did not appear to cause any bilayer disruption, indicating that decreased fluidity of the membrane somehow enhanced the interaction between the bilayer and A β . Furthermore, although different types of A β (1–40) aggregates were observed in these studies, these aggregates were formed over the same incubation time in A β (1–40) solution without the presence of the bilayers, indicating that this aggregation was somehow facilitated by the presence of such bilayers.

4.2.3. Other Factors Affecting A β Aggregation

An important theme in the studies of A β fibrillogenesis is the possibility to control it with various (bio)chemical agents such as apolipoproteins, chemical chaperones, and antibodies. Suspension of fibrillogenesis by appropriately chosen agents is considered as one potential preventative/therapeutic strategy. In recent years, AFM has been playing an increasingly important role in the studies of such interactions. Without definitive knowledge of the A β form that is the primary culprit in AD pathology, the possibility that such a strategy may actually stabilize the toxic species of A β is a potential issue. Such a scenario makes it increasingly important that methods of preparing stable forms of A β at different stages of aggregation be developed for studies aimed at elucidating the most toxic species along its aggregation pathway.

Clusterin (apolipoprotein J), which is an abundant lipoprotein found in the central nervous system and has been shown to affect A β toxicity, was found to promote the formation of small globular structures (or ADDLs) in solutions of A β (1–42) (Lambert *et al.*, 1998). Clusterin has been known to suppress A β fibrillogenesis in solution and to promote the formation of neurotoxic globular structures (ADDLs). AFM analysis of these structures revealed that their height ranged from 4.8–5.7 nm; whereas, their volumes did not exceed the value expected for ~17–42-kDa compact protein globules. Because the latter size was too small to have clusterin (~70–75 kDa) associated with these aggregates, they were most likely comprised solely of A β . In the absence of clusterin, similar ADDLs were found

to be a dominant form only in solutions incubated at reduced temperatures (4–8°C). Such observations appear to be consistent with the notion that ADDLs represent the early stages of A β aggregation, and that their evolution into fibrillar species can be averted by various factors. Such yet unidentified factors appear to be present in clusterin-free cell culture media, where ADDLs were observed to be the predominant form when aggregation was carried out at 37°C (i.e., under conditions which usually accelerate fibrillogenesis). The ADDLs formed under these various conditions were indistinguishable from each other and stable for periods of up to 24 hours.

The impact of chemical chaperones or organic osmolytes on the aggregation pathway of A β (1–40) was studied using *in situ* AFM (Yang *et al.*, 1999). When trimethylamine N-oxide (TMAO) or glycerol were added to A β (1–40) samples at conditions that were verified by circular dichroism to completely convert a random coil to a β -sheet structure, a mixture of protofibrils exhibiting 10 nm periodicity and small elliptical aggregates was observed. Elliptical aggregates, which represented the majority of species, were ~5 nm tall, ~1.5 wide, and ~6 nm long. Given that the volume of a single A β peptide is of the order of 10,000 Å³, the estimated volume (~45,500 Å³) would be equivalent to tetramers or pentamers of A β (without taking into account the contribution of the AFM tip). The dimensions of these aggregates were also close to those of ADDLs described previously.

Atomic force microscopy has also been used to explore the effects of different anti-A β antibodies on the early stages of fibrillization (Figure 12.3-5) (Legleiter *et al.*, 2004). The m3D6 antibody, which binds to the N-terminus of A β , and the m266.2 antibody, which binds to the center portion of A β , were incubated with A β (1–42), and fibril growth was monitored over several days using AFM. It was found that in the presence of m3D6 the fibrils formed at a slower rate and tended to be shorter than those formed in control solutions. Fibrils formed in the presence of m3D6 were also decorated on their periphery by a halo of antibodies that was not present in control studies (Figure 12.3-5B). In contrast, m266.2 completely prevented the formation of fibrils over the time course of the experiment, and only globular species were observed (Figure 12.3-5C).

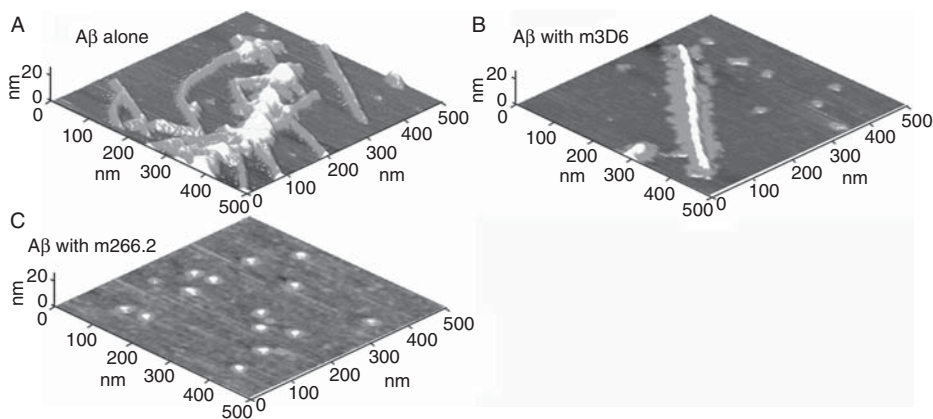


Figure 12.3-5. Atomic force microscopy images taken on the fifth day of incubation clearly show the impact of antibodies binding to the different portions of the peptide on A β aggregation. Although A β alone forms numerous extended fibrils (A), in the presence of the m3D6 antibody, which binds to the N-terminus, the fibrils are less numerous and their periphery is surrounded by a “halo” of antibodies (B). In great contrast, no fibrils are formed in the presence of the m266 antibody, which binds to the central portion of the peptide, and only compact, globular aggregates are observed (C).

5. Studies of α -Syn Aggregation and Morphology

α -Synuclein is a 140-amino acid long protein of relatively high abundance in the brain and primarily unfolded in its native state. Its physiological function is as of yet unknown. The ordered aggregation of α -syn in intracellular inclusions or Lewy bodies is a key feature in the common neurodegenerative disorder known as PD (Spillantini *et al.*, 1998). Early-onset PD has been associated with two point mutations in the gene encoding α -syn (A53T and A30P) (Polymeropoulos *et al.*, 1997; Kruger *et al.*, 1998), suggesting potential importance of α -syn aggregation in PD. As in the case of A β and AD, AFM has emerged as an important characterization tool in the study of α -syn aggregation associated with PD.

5.1. α -Syn Aggregation and Fibrillization

5.1.1. Fibril Assembly Pathway

The aggregation pathway for α -syn has been found to be strikingly similar to that of A β . Spherical aggregates ~3–4 nm in height were observed during the initial stages of fibrillogenesis. These spherical aggregates later gave rise to protofilaments with observed heights of 3.8 ± 0.6 nm (Khurana *et al.*, 2003). These protofilaments further aggregated and twisted to form periodic protofibrils with an average height of 6.5 ± 0.6 nm. Mature periodic fibrils with an average height of 9.8 ± 1.2 nm were then comprised of twisting protofibrils. Other morphologies have also been observed. Spherical aggregates of an equimolar mixture of A53T with wild type (WT) collected from the void volume from gel filtrated chromatography of the supernatant of sedimented samples were shown to form annular or ring-like structures at 4°C (Conway *et al.*, 2000b). The original spherical species ranged in height from 2–6 nm, but no elongated fibrillar structures were observed. After 72 hours of incubation, two types of annular species were observed: circular and elliptical. The circular species possessed diameters of 35–55 nm; the elliptical structures dimensions were observed to be in the range of 35–55 nm by 65–130 nm. Both types of annular structures had average heights of 2–4 nm and displayed periodicities.

5.1.2. The Exploration of α -Syn Sequence: Studies of Mutants and Fragments

When WT, A53T, and A30P α -syn were incubated at concentrations of 100–300 μ M, mature fibrils of 8–10 nm in height were observed for all samples along with individual filaments (~5 nm in height) and spherical aggregates (~4 nm in height) (Figure 12.3-6) (Conway *et al.*, 2000a). These fibrils often displayed the twisted helical morphology reminiscent of intertwined protofibrils with periodicities of 45, 65, and 95 nm, especially in samples of A53T. Whereas WT and A53T fibrils formed at 300 μ M were linear (Figure 12.3-6B), A30P fibrils possessed a meandering, almost “sinusoidal” structure (Figure 12.3-6C). At lower concentrations, however, there were no observable differences between mature fibrils of all three variants. Interestingly, the observed dimensions of fibrils formed in solution compared favorably to those observed for fibrils obtained from actual PD brain section Lewy bodies.

AFM observations of WT, A53T, and A30P also revealed the formation of other morphologies including annular, nonfibrillar structures (Figure 12.3-7) (Ding *et al.*, 2002). Observations of initial stages of aggregation of WT and A30P revealed spherical aggregates. WT aggregates were on average 4.2 nm tall, whereas the average height for A30P aggregates was 2.7 nm. Spherical aggregates were also observed for A53T at early stages of aggregation. They appeared to be a mixture of the two types of aggregates observed for the other two variants of α -syn (2.7 and 4.2 nm). The spherical 2.7-nm tall aggregates appeared to be the more “mature” form of spherical aggregates because the population of 4.2-nm tall aggregates of WT α -syn eventually diminished at the expense of the smaller

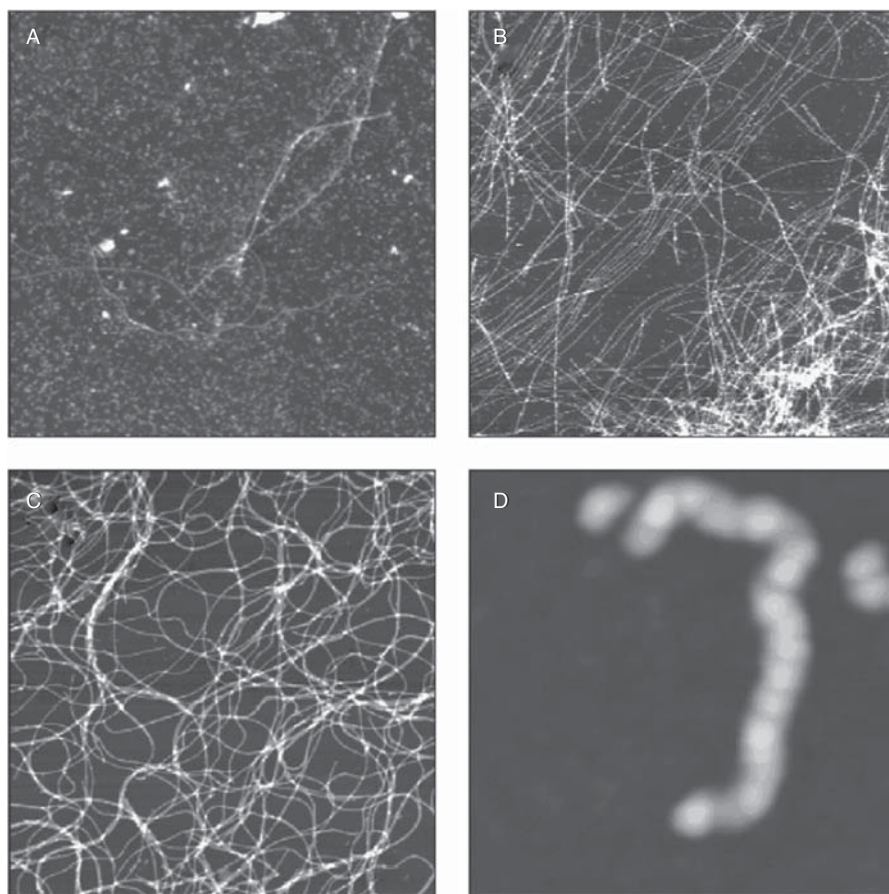


Figure 12.3-6. Fibrils formed by different α -syn mutants following incubation in solution: (A) 300 μ M WT, 4 months; (B) 100 μ M A53T, 1 month; (C) 300 μ M A30P, 4 months; (D) 50 μ M A30P, 4 months. Image sizes for (A–C) are $5 \times 5 \mu\text{m}^2$, and $250 \times 250 \text{ nm}^2$ for image (D). (Reprinted with permission from Conway *et al.*, 2000a. Copyright 2000 from the American Chemical Society.)

form. When incubated at 4°C, these spherical aggregates gave way to annular structures. Two types of WT annular structures were observed: WT1 (32–96 nm in diameter) and WT2 (100–180 nm in diameter). The WT1 structures had an average height of ~3 nm, which corresponds well to the height of the observed spherical precursors. Regions with heights of ~7 nm and structural elements of mature amyloid fibrils were observed in WT2 structures. A53T formed annular structures with diameters of only ~10 nm. A30P formed annular structures after 48 hours when incubated at 37°C, but they were never observed for incubations held at 4°C. These annular structures had an average diameter of ~55 nm and a height of ~2.2 nm. Such annular structures may play a role in α -syn-related toxicity by forming membrane-spanning pores.

Like the A53T mutation, murine α -syn contains a threonine residue at position 53, but it differs from the WT by seven residues. Despite this difference, fibrils and fibrillar bundles were still observed

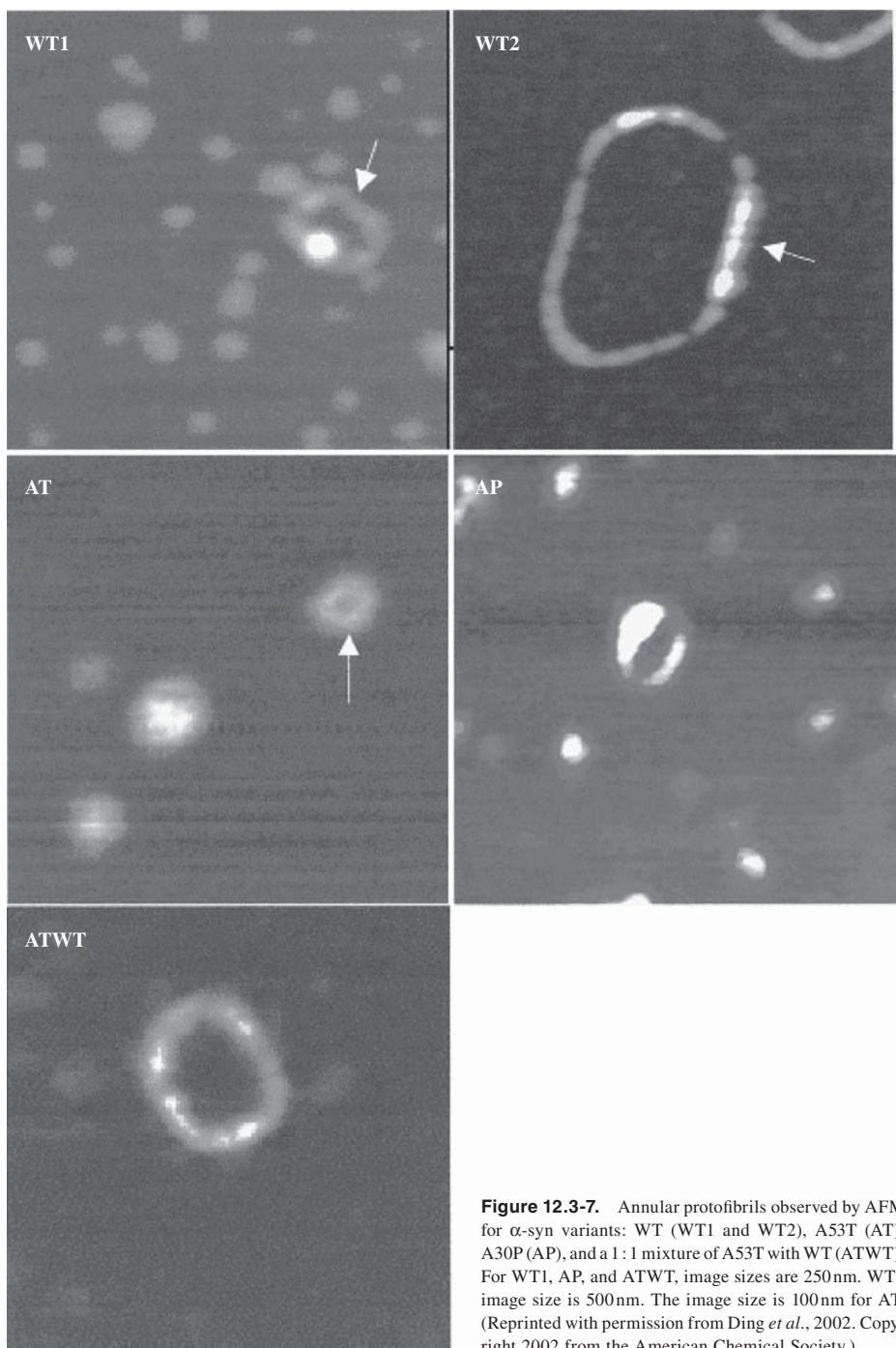


Figure 12.3-7. Annular protofibrils observed by AFM for α -syn variants: WT (WT1 and WT2), A53T (AT), A30P (AP), and a 1 : 1 mixture of A53T with WT (ATWT). For WT1, AP, and ATWT, image sizes are 250nm. WT2 image size is 500nm. The image size is 100nm for AT. (Reprinted with permission from Ding *et al.*, 2002. Copyright 2002 from the American Chemical Society.)

using AFM (Rochet *et al.*, 2000). These fibrils were straight, rigid, and unbranched, with lengths of 0.1–3 μm and heights of ~ 7.6 nm, which is very similar to fibrils observed for human variants of α -syn. Twisted fibrils consisting of two subfilaments were also observed, with periodicities that varied from 55–68 nm; however, the periodicity remained constant within the same fibril.

The role of different amino acids and peptide sequences was explored with AFM by incubating various mutants and portions of α -syn (Du *et al.*, 2003). WT α -syn formed fibrils within 6 days, but charged mutants (G68E, G68R, V66R, V70R/V71R, and V74R) were unable to fibrillize for up to 15 days. G68E, V70R/V71R, and V74R all formed small oligomers that had heights of ~ 8 nm. Mutant G68A actually accelerated aggregation. α -Syn, with a deletion of 66–74 ($\Delta 66$ –74), did not form fibrils, even with up to 6 weeks of incubation. α -Syn1–74 exhibited significantly accelerated fibrillization, as fibrils were observed after 2 days. Fibril formation was slower for α -syn1–70 compared to WT α -syn. A mutant with amino acids 71–74 deleted ($\Delta 71$ –74) was only able to form oligomers. These studies indicated that a nine residue peptide motif of $^{66}\text{VGGAVVTGV}^{74}$ plays an important role in the fibrillization of α -syn.

5.2. Factors Modulating α -Syn Aggregation

5.2.1. The Role of pH

In situ AFM observations were performed on WT α -syn aggregates to determine the effect of pH on aggregate morphology (Hoyer *et al.*, 2002). At pH of 6.0 and 7.0, α -syn adopted a fibrillar structure with various lengths ranging from 100–1000 nm and height of ~ 11 nm. However, these fibrils differed in morphology; whereas fibrils formed at pH of 7.0 were smooth, twisted morphology (periodicity of 40–120 nm) dominated in fibrils formed at pH of 6.0. Samples imaged at pH of 5.0 and 4.0 consisted mostly of large, irregularly shaped, amorphous aggregates. The sample incubated at pH 5.0 consisted of smaller aggregates with some adjoining fibrils observed. At pH 4.0, the sample was all amorphous once steady state was reached; however, short fibril-like structures were observed at the beginning of the growth phase (~ 20 min), as determined by ANS and ThT fluorescence. These structures were ~ 50 nm in length and ~ 8 nm in height.

5.2.2. Interactions of α -Syn with Lipids

In situ AFM was used to study the effect of α -syn on lipid bilayers consisting of 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC)/1-palmitoyl-2-oleoyl phosphatidylserine (POPS) (Figure 12.3-8) (Jo *et al.*, 2000). WT α -syn disrupted the bilayers at a concentration of 0.1 mg/ml by the formation of small bilayer holes that expanded over the course of several hours. Closer inspection of the disrupted bilayer revealed both small aggregates (~ 2 –3 nm in width and ~ 2 nm thick) and putative fibrils formed on the exposed mica. The small aggregates were most likely comprised of α -syn or α -syn/lipid, but were unlikely small lipid artifacts, because they displayed distinct contrast in phase images. The A53T mutant was observed to disrupt bilayers, but at a slower rate. The A53T aggregates observed on the exposed regions of mica differed in size from those observed for WT.

When brain-derived lipid membranes were exposed to spherical or protofibrillar α -syn aggregates, there seemed to be no effect on the membranes; however, ring-like structures of α -syn were observed when vesicles, which served as precursors to membranes, were pretreated with the spherical aggregates described earlier (Ding *et al.*, 2002). These structures were not abundant (four structures found in $77 \mu\text{m}^2$) and were all smaller than 30 nm in diameter. In another study with different phospholipids, AFM was used in a similar way to confirm disruption of vesicular membranes by α -syn (Volles *et al.*, 2001). β -Sheet-rich, membrane-spanning pores formed from α -syn membrane-bound annular structures are strikingly similar to protein toxins (i.e., α -hemolysin, perfringolysin, and

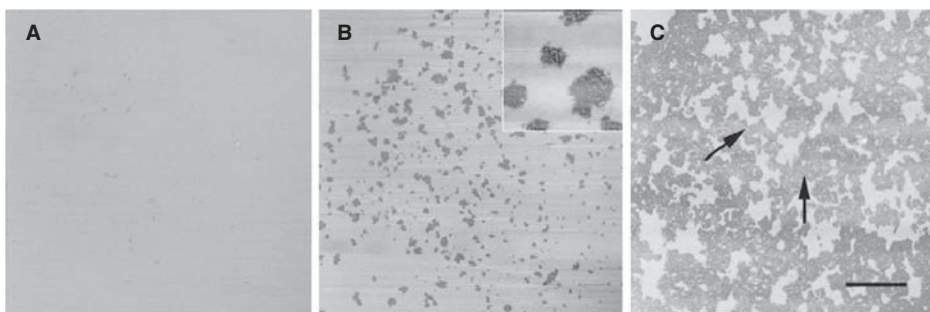


Figure 12.3-8. Disruption of a lipid bilayer by WT α -syn visualized by AFM. (A) Intact bilayer of equimolar POPC/POPS on mica. (B–C) Images acquired following 30 minutes (B) and 9 hours (C) after the addition of 0.1 mg/ml of α -syn. Inset in (B) shows α -syn aggregates visible on the exposed mica. (scale bar represents 200 nm). (From Jo *et al.*, Journal of Biological Chemistry. Copyright 2000 by the American Society for Biochemistry & Molecular Biology. Reproduced with permission from the American Society for Biochemistry & Molecular Biology in the format Textbook via the Copyright Clearance Center.)

anthrax protective antigen), suggesting that such annular structures may play an important role in neurotoxicity.

α -Synuclein has also been observed on membranes fused from large unilamellar vesicles (Zhu *et al.*, 2003). When 0.3 μ g/ml of α -syn was added to PA/PC stacked bilayers, aggregates of α -syn with heights of 2–6 nm were observed. After 1.5 hours, these aggregates disappeared as they incorporated into the bilayer, which was accompanied by the appearance of circular bilayer defects. These defects covered 26% of the surface and were of different heights, indicating that α -syn was destroying the membrane layer by layer. After 2 hours, 58% of the surface was exposed, and this increased to 79% after 4 hours. Despite this membrane disruption, no fibrils were observed. Preformed α -syn fibrils were incubated on these planar lipid bilayers, and these fibrils were clearly observable on the bilayer surface with heights of 3.2–6.0 nm. These fibrils normally would have heights of $\sim$ 7.8 nm, indicating insertion or embedding into the bilayer. After 0.5 hours of incubation with fibrils, 80% of the surface was exposed, and only bare mica was observed after 2 hours. Different mixtures of lipids in the bilayer were also studied. PA/PE and PG/PE planar lipid bilayers were both disrupted by α -syn in 4–16 hours. PA/PE was disrupted more rapidly than PG/PE. The disrupted areas of PG/PE appeared more ordered, as linear defects formed before the final breakdown of the membrane.

5.2.3. Other Factors Affecting α -Syn Aggregation

To explore the possibility that molecular crowding due to an increase in the cytoplasmic protein concentration could accelerate fibrillization, WT α -syn was incubated with poly(ethyleneglycol) (PEG) to simulate molecular crowding (Shtilerman *et al.*, 2002). PEG was used due to its biologically inert nature. The aggregation of WT α -syn was shown to be accelerated by several methods, including ThT fluorescence and size exclusion chromatography. Atomic force microscopy was used to verify that the α -syn aggregate structures were not altered in comparison with those formed in PEG-free solutions. These structures did not differ when imaged, and aggregation proceeded via a similar, but accelerated, progression of structures. Spherical aggregates and protofibrils (3–4 nm in height) formed before mature fibrils (heights of $\sim$ 7 nm). Mature fibrils and annular aggregates were found to coexist in molecularly crowded incubations.

The aggregation of α -syn in the presence of cellular polyamines putrescine, spermidine, and spermine was studied by various techniques, including AFM (Figure 12.3-9) (Antony *et al.*, 2003).

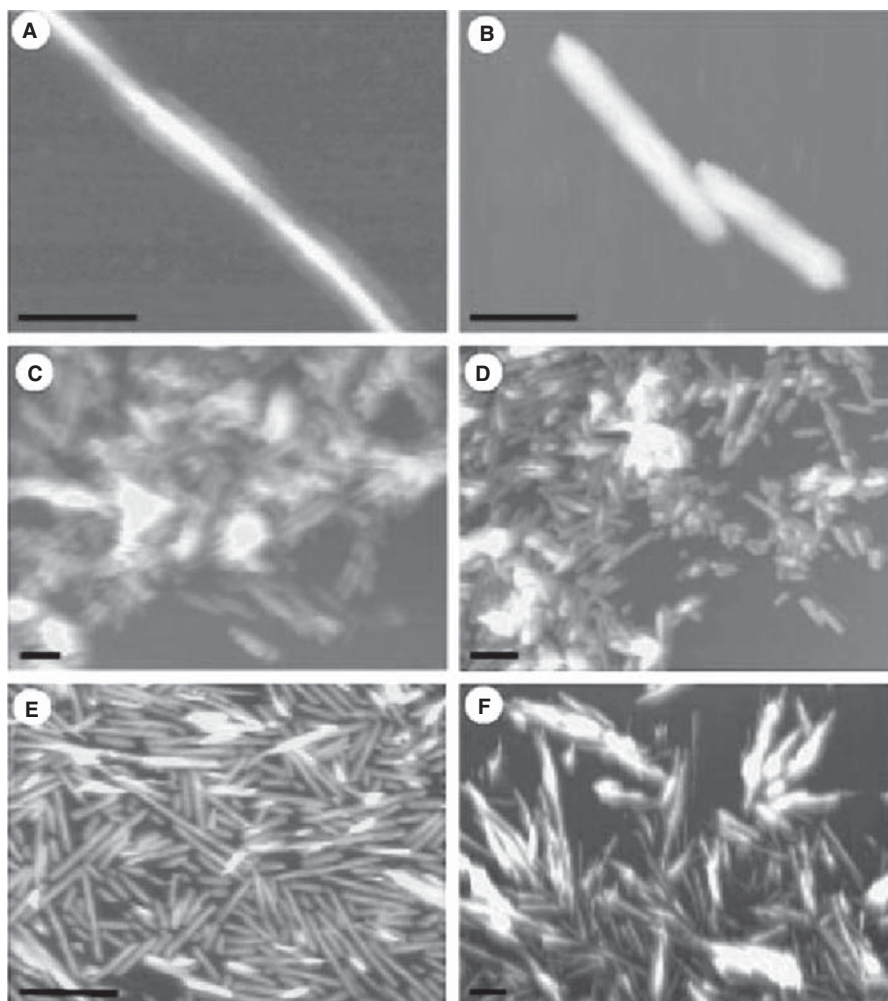


Figure 12.3-9. AFM images of α -syn aggregated in the absence and presence of polyamines: (A) α -Synuclein alone (48 hours); (B) with 1 mM spermidine (4 hours); (C) with 100 μ M spermine (4 hours); (D) with 1 mM spermidine (24 hours); (E) with 5 mM putrescine (24 hours); and (F) with 5 mM putrescine (4 days). Scale bars: (A) and (B) 100 nm, (C–F) 200 nm. (From Antony *et al.* Journal of Biological Chemistry. Copyright 2003 by the American Society for Biochemistry & Molecular Biology. Reproduced with permission from the American Society for Biochemistry & Molecular Biology in the format Textbook via the Copyright Clearance Center.)

Polyamines are organic polycations that occur naturally and are involved in several cellular functions. Polyamine levels in the substantia nigra of diseased human brains are not lowered, indicating that their regulation is well maintained in degenerating cells (Vivo *et al.*, 2001). In the absence of polyamines, α -syn aggregated into fibrils of height $\sim$ 12 nm within 48 hours (Figure 12.3-9A) (Antony *et al.*, 2003). In contrast, various protein aggregates were seen in samples incubated with polyamines for up to 4 hours. In the presence of spermidine, short, isolated fibrils were formed with heights of

~12 nm (Figure 12.3-9B). However, in the presence of spermine, only large amorphous aggregates with diffuse structures were observed. These large amorphous aggregates formed in the presence of 1 mM of spermidine (Figure 12.3-9D), but this required 24 hours of incubation. Large aggregates consisting of clearly distinguishable short fibrils were present at both 4 hours (Figure 12.3-9E) and 24 hours (Figure 12.3-9F) of incubation for α -syn in the presence of putrescine.

6. Studies of Other Amyloid-Forming Peptides

There are several other amyloid forming proteins that are associated with different conformational diseases, and AFM has been used in studying many of them including: the tumor suppressor p53 core domain (Ishimaru *et al.*, 2003); various prion proteins like Sup53 (Serio *et al.*, 2000), the Y145Stop variant of human prion protein (Kundu *et al.*, 2003), and the yeast prion protein Ure2p (Bousset *et al.*, 2002); other proteins associated with AD such as Tau (Tseng *et al.*, 1999), lithostathine (Gregoire *et al.*, 2001), and paired helical filaments (Moreno-Herero *et al.*, 2004); islet amyloid polypeptide (Kayed *et al.*, 1999; Tenidis *et al.*, 2000); β 2-microglobulin (Kad *et al.*, 2001, 2003); and immunoglobulin light chains (Ionescu-Zanetti *et al.*, 1999; Khurana, Ritu *et al.*, 2001; Zhu *et al.*, 2002). One particular study of immunoglobulin light chains demonstrated the importance of surfaces in protein aggregation and fibrillization (Figure 12.3-10) (Zhu *et al.*, 2002). Small pieces of

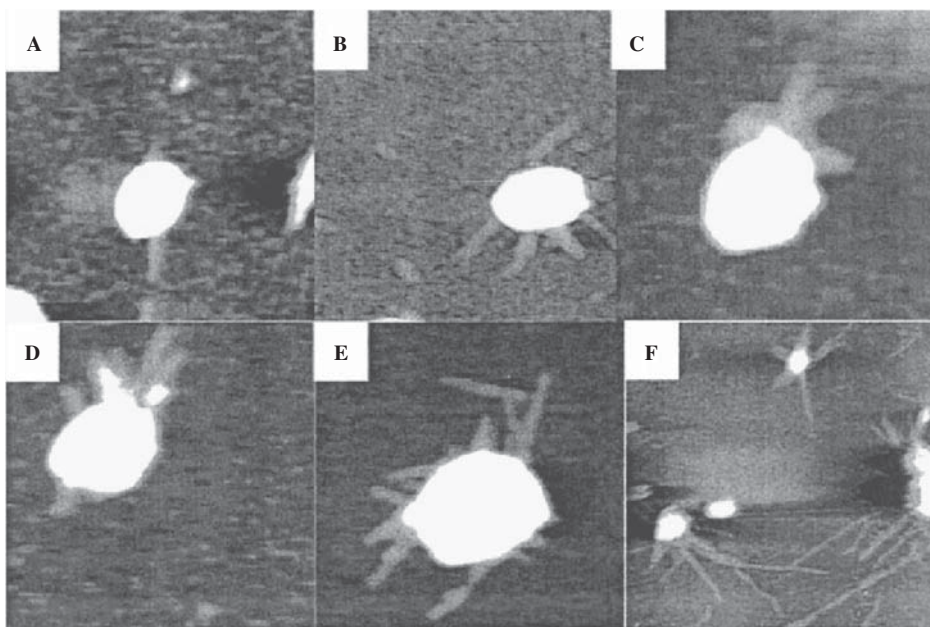


Figure 12.3-10. Surface-catalyzed fibrillization of SMA antibodies. After 12–16 hours of incubation, central cores were observed with one (A), four (B and C), six (D), or nine (E) protofibrils radiating out from them (1 μ m images). After 6 days, mature fibrils resulted from further elongation of protofibrils [(F), scale of 5 μ m]. Incubation conditions were pH of 5.0, 50 μ g/ml of SMA antibody at 37°C. (From Zhu *et al.* Journal of Biological Chemistry. Copyright 2002 by the American Society for Biochemistry & Molecular Biology. Reproduced with permission from the American Society for Biochemistry & Molecular Biology in the format Textbook via the Copyright Clearance Center.)

mica were incubated in solutions containing a recombinant amyloidogenic light-chain variable domain of smooth muscle actin (SMA) antibody, under conditions in which fibrils normally did not form (i.e., low concentration and no agitation). At short times, amorphous aggregates appeared on mica, and fibrils were observed within 10 hours. Fibrils were not formed in the solution within the same time frame. The fibrils on the surface of mica grew from the amorphous aggregates and the assemblies of oligomers present on mica.

7. Conclusions

Atomic force microscopy has proven to be a useful tool in studying the aggregation of peptides associated with various conformational diseases under a wide variety of conditions. Due to its unique ability to follow in time three-dimensional nanoscale surface maps without extensive sample preparations and under nearly physiological conditions, it can provide information that is not easily accessible by other methods. It is particularly useful in studying the importance of surfaces in the processes leading to pathological peptide assembly and the structural consequences of the addition of modulating factors on such aggregation. Biological relevance of *in situ* AFM experiments is further enhanced by the fact that supported lipid bilayers and cells can be employed as substrates under physiological buffer conditions. Used in conjunction with other techniques, AFM has become an invaluable tool providing useful information about disease-related protein aggregation. The use of AFM as a complementary technique that can distinguish morphology and size of protein aggregates in conjunction with other techniques can aid in determining toxic species involved in various conformational diseases. A particularly attractive combination of techniques would be *in situ* AFM on bilayer substrates with simultaneous fluorescence imaging that can track the fate of labeled proteins.

8. Abbreviations

A β	β -Amyloid peptide
AD	Alzheimer's disease
ADDL	Amyloid-derived diffusible ligand
α -syn	α -Synuclein
AFM	Atomic force microscopy
APP	Amyloid precursor protein
PEG	Poly(ethyleneglycol)
PD	Parkinson's disease
POPC	1-palmitoyl-2-oleoyl phosphatidylcholine
POPS	1-palmitoyl-2-oleoyl phosphatidylserine
ThT	Thioflavin T
TMAO	Trimethylamine N-oxide

References

- Antony, T., Hoyer, W., Cherny, D., Heim, G., Jovin, T.M., and Subramaniam, V. (2003). Cellular polyamines promote the aggregation of α -synuclein. *J. Biol. Chem.* 278:3235–3240.
- Bhatia, R., Lin, H., and Lal, R. (2000). Fresh and nonfibrillar amyloid β protein(1–42) induces rapid cellular degeneration in aged human fibroblasts: evidence for A β P-channel-mediated cellular toxicity. *FASEB J.* 14:1233–1243.

- Binnig, G., Quate, C.F., and Gerber, C. (1986). Atomic force microscope. *Phys. Rev. Lett.* 56:930–933.
- Blackley, H.K.L., Patel, N., Davies, M.C., Roberts, C.J., Tendler, S.J.B., Wilkinson, M.J., and Williams, P.M. (1999). Morphological development of β (1–40) amyloid fibrils. *Exp. Neurol.* 158:437–443.
- Blackley, H.K.L., Sanders, G.H.W., Davies, M.C., Roberts, C.J., Tendler, S.J.B., and Wilkinson, M.J. (2000). *In-situ* atomic force microscopy study of β -amyloid fibrillization. *J. Mol. Biol.* 298:833–840.
- Bousset, L., Thomson, N.H., Radford, S.E., and Melki, R. (2002). The yeast prion Ure2p retains its native α -helical conformation upon assembly into protein fibrils *in vitro*. *EMBO J.* 21:2903–2911.
- Conway, K.A., Harper, J.D. and Lansbury, P.T., Jr. (2000a). Fibrils formed in vitro from α -synuclein and two mutant forms linked to Parkinson's disease are typical amyloid. *Biochemistry* 39:2552–2563.
- Conway, K.A., Lee, S.-J., Rochet, J.-C., Ding, T.T., Williamson, R.E., and Lansbury, P.T., Jr. (2000b). Acceleration of oligomerization, not fibrillization, is a shared property of both α -synuclein mutations linked to early-onset Parkinson's disease: Implications for pathogenesis and therapy. *Proc. Natl. Acad. Sci. USA.* 97:571–576.
- Crowther, D.C. (2002). Familial conformational diseases and dementias. *Hum. Mutat.* 20:1–14.
- Ding, T.T., Lee, S.-J., Rochet, J.-C., and Lansbury, P.T., Jr. (2002). Annular α -synuclein protofibrils are produced when spherical protofibrils are incubated in solution or bound to brain-derived membranes. *Biochemistry* 41:10209–10217.
- Du, H.-N., Tang, L., Luo, X.-Y., Li, H.-T., Hu, J., Zhou, J.-W., and Hu, H.-Y. (2003). A peptide motif consisting of Glycine, Alanine, and Valine is required for the fibrillization and cytotoxicity of human α -synuclein. *Biochemistry* 42:8870–8878.
- Goldsbury, C., Kistler, J., Aebi, U., Arvinte, T., and Cooper, G.J.S. (1999). Watching amyloid fibrils grow by time-lapse atomic force microscopy. *J. Mol. Biol.* 285:33–39.
- Gregoire, C., Marco, S., Thimonier, J., Duplan, L., Laurine, E., Chauvin, J.-P., Michel, B., Peyrot, V., and Verdier, J.-M. (2001). Three-dimensional structure of the lithostathine protofibril, a protein involved in Alzheimer's disease. *EMBO J.* 20:3313–3321.
- Hansma, P.K., Cleveland, J.P., Radmacher, M., Walters, D.A., Hillner, P.E., Bezanailla, M., Fritz, M., Vie, D., Hansma, H.G., Prater, C.B., Massie, J., Fukunaga, L., Gurley, J., and Elings, V. (1994). Tapping mode atomic force microscopy in liquids. *Appl. Phys. Lett.* 64:1738–1740.
- Harper, J.D., Lieber, C.M., and Lansbury, P.T., Jr. (1997a). Atomic force microscopy imaging of seeded fibril formation and fibril branching by the Alzheimer's disease amyloid- β protein. *Chem. Biol.* 4:951–959.
- Harper, J.D., Wong, S.S., Lieber, C.M., and Lansbury, P.T., Jr. (1997b). Observation of metastable A β amyloid protofibrils by atomic force microscopy. *Chem. Biol.* 4:119–125.
- Hoyer, W., Antony, T., Cherny, D., Heim, G., Jovin, T.M., and Subramaniam, V. (2002). Dependence of α -synuclein aggregate morphology on solution conditions. *J. Mol. Biol.* 322:383–393.
- Ionescu-Zanetti, C., Khurana, R., Gillespie, J.R., Petrick, J.S., Trabachino, L.C., Minert, L.J., Carter, S.A., and Fink, A.L. (1999). Monitoring the assembly of Ig light-chain amyloid fibrils by atomic force microscopy. *Proc. Nat. Acad. Sci. USA.* 96:13175–13179.
- Ishimaru, D., Andrade, L.R., Teixeira, L.S.P., Quesado, P., Maiolino, L.M., Lopez, P.M., Cordeiro, Y., Costa, L.T., Heckl, W.M., Weissmuller, G., Foguel, D., and Silva, J.L. (2003). Fibrillar aggregates of the tumor suppressor p53 core domain. *Biochemistry* 42:9022–9027.
- Jarrett, J.T., Berger, E.P., and Lansbury, P.T. (1993). The carboxy terminus of the amyloid protein is critical for the seeding of amyloid formation: Implications for the pathogenesis of Alzheimer's disease. *Biochemistry* 32:4693–4697.
- Jo, E., McLaurin, J., Yip, C.M., St. George-Hyslop, P., and Fraser, P. E. (2000). α -Synuclein membrane interactions and lipid specificity. *J. Biol. Chem.* 275:34328–34334.
- Kad, N.M., Thomson, N.H., Smith, D.P., Smith, D.A., Radford, S.E. (2001). β_2 -Microglobulin and its deamidated variant, N17D form amyloid fibrils with a range of morphologies *in vitro*. *J. Mol. Biol.* 313:559–571.
- Kad, N.M., Myers, S.L., Smith, D.P., Smith, D.A., Radford, S.E., and Thomson, N.H. (2003). Hierarchical assembly of β_2 -microglobulin amyloid in vitro revealed by atomic force microscopy. *J. Mol. Biol.* 330:785–797.
- Kayed, R., Bernhagen, J., Greenfield, N., Sweimeh, K., Brunner, H., Voelter, W., and Kapurniotu, A. (1999). Conformational transitions of islet amyloid polypeptide (IAPP) in amyloid formation in vitro. *J. Mol. Biol.* 287:781–796.
- Kelly, J.W. (1998). The alternative conformations of amyloidogenic proteins and their multi-step assembly pathways. *Curr. Opin. Struct. Biol.* 8:101–106.
- Khurana, R., Gillespie, J.R., Talapatra, A., Minert, L.J., Ionescu-Zanetti, C., Millett, I., and Fink, A.L. (2001). Partially folded intermediates as critical precursors of light chain amyloid fibrils and amorphous aggregates. *Biochemistry* 40:3525–3535.
- Khurana, R., Ionescu-Zanetti, C., Pope, M., Li, J., Nielson, L., Ramirez-Alvarado, M., Regan, L., Fink, A.L., and Carter, S.A. (2003). A general model for amyloid fibril assembly based on morphological studies using atomic force microscopy. *Biophys. J.* 85:1135–1144.

- Kowalewski, T., and Holtzman, D.M. (1999). *In situ* atomic force microscopy study of Alzheimer's β -amyloid peptide on different substrates: new insights into mechanism of β -sheet formation. *Proc. Natl. Acad. Sci. USA* 96:3688–3693.
- Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J.T., Schols, L., and Riess, O. (1998). Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nat. Genet.* 18:106–108.
- Kundu, B., Maiti, N.R., Jones, E.M., Surewicz, K.A., Vanik, D.L., and Surewicz, W.K. (2003). Nucleation-dependent conformational conversion of the Y145Stop variant of human prion protein: structural clues for prion propagation. *Proc. Natl. Acad. Sci. USA* 100:12069–12074.
- Lambert, M.P., Barlow, A.K., Chromy, B.A., Edwards, C., Freed, R., Liosatos, M., Morgan, T.E., Rozovsky, I., Trommer, B., Viola, K.L., Wals, P., Zhang, C., Finch, C.E., Krafft, G.A., and Klein, W.L. (1998). Diffusible, nonfibrillar ligands derived from $A\beta_{1-42}$ are potent central nervous system neurotoxins. *Proc. Natl. Acad. Sci. USA* 95:6448–6453.
- Lansbury, P.T., Jr. (1996). A reductionist view of Alzheimer's disease. *Acc. Chem. Res.* 29:317–321.
- Lantz, M., Liu, Y.Z., Cui, X.D., Tokumoto, H., and Lindsay, S.M. (1999). Dynamic force microscopy in fluid. *Surface Interface Anal.* 27:354–360.
- Legleiter, J., Czilli, D.L., Gitter, B., DeMattos, R.B., Holtzman, D.M., and Kowalewski, T. (2004). Effect of different anti- $A\beta$ antibodies on $A\beta$ fibrillogenesis as assessed by atomic force microscopy. *J. Mol. Biol.* 335:997–1006.
- Lin, H., Zhu, Y.J., and Lal, R. (1999). Amyloid β protein (1–40) forms calcium-permeable, Zn^{2+} -sensitive channel in reconstituted lipid vesicles. *Biochemistry* 38:11189–11196.
- Lin, H., Bhatia, R., and Lal, R. (2001). Amyloid β protein forms ion channels: implications for Alzheimer's disease pathophysiology. *FASEB J.* 15:2433–2444.
- Liu, R., McAllister, C., Lyubchenko, Y., and Sierks, M.R. (2004). Residues 17–20 and 30–35 of beta-amyloid play critical roles in aggregation. *J. Neurol. Res.* 75:162–171.
- Malkin, A.J., Kuznetsov, Y.G., Glantz, W., and McPherson, A. (1996). Atomic force microscopy studies of surface morphology and growth kinetics in thaumatin crystallization. *Proteins Struct. Funct. Genet.* 24:247–252.
- Moreno-Herero, F., Perez, M., Baro, A.M., and Avila, J. (2004). Characterization by atomic force microscopy of alzheimer paired helical filaments under physiological conditions. *Biophys. J.* 86:517–525.
- Nichols, M.R., Moss, M.A., Reed, D.K., Lin, W.-H., Mukhopadhyay, R., Hoh, J.H., and Rosenberry, T.L. (2002). Growth of β -amyloid(1–40) protofibrils by monomer elongation and lateral association. characterization of distinct products by light scattering and atomic force microscopy. *Biochemistry* 41:6115–6127.
- Polymopoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E.S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W.G., Lazzarini, A.M., Duvoisin, R.C., Di Iorio, G., Golbe, L.I., and Nussbaum, R.L. (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science*. 276:2045–2047.
- Putman, C.A.J., van der Werf, K.O., De Grooth, B.G., Van Hulst, N.F., and Greve, J. (1994). Tapping mode atomic force microscopy in liquid. *Appl. Phys. Lett.* 64:2454–2456.
- Rhee, S.K., Quist, A.P., and Lal, R. (1998). Amyloid β protein-(1–42) forms calcium-permeable, Zn^{2+} -sensitive channel. *J. Biol. Chem.* 273:13379–13382.
- Rochet, J.-C., Conway, K.A., and Lansbury, P.T., Jr. (2000). Inhibition of fibrillization and accumulation of prefibrillar oligomers in mixtures of human and mouse α -synuclein. *Biochemistry* 39:10619–10626.
- Roher, A.E., Chaney, M.O., Kuo, Y.-M., Webster, S.D., Stine, W.B., Jr., Haverkamp, L.J., Woods, A.S., Cotter, R.J., Tuohy, J.M., Krafft, G.A., Bonnell, B.S., and Emmerling, M.R. (1996). Morphology and toxicity of $A\beta$ -(1–42) dimer derived from neuritic and vascular amyloid deposits of Alzheimer's disease. *J. Biol. Chem.* 271:20631–20635.
- Selkoe, D.J. (2001). Alzheimer's disease: genes, proteins, and therapy. *Physiol. Rev.* 81:741–766.
- Serio, T.R., Cashikar, A.G., Kowal, A.S., Sawicki, G.J., Moslehi, J.J., Serpell, L., Arnsdorf, M.F., and Lindquist, S.L. (2000). Nucleated conformational conversion and the replication of conformational information by a prion determinant. *Science* 289:1317–1321.
- Shitlerman, M.D., Ding, T.T., and Lansbury, P.T., Jr. (2002). molecular crowding accelerates fibrillization of α -synuclein: could an increase in the cytoplasmic protein concentration induce Parkinson's disease? *Biochemistry* 41:3855–3860.
- Smith, M.A. (1998). Alzheimer disease. *Int. Rev. Neurobiol.* 42:1–54.
- Spillantini, M.G., Crowther, R.A., Jakes, R., Hasegawa, M., and Goedert, M. (1998). α -Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. *Proc. Natl. Acad. Sci. USA* 95:6469–6473.
- Stine, W.B., Jr., Dahlgren, K.N., Krafft, G.A., and LaDu, M.J. (2003). *In vitro* characterization of conditions for amyloid- β peptide oligomerization and fibrillogenesis. *J. Biol. Chem.* 278:11612–11622.
- Stine, W.B., Jr., Synder, S.W., Lador, U.S., Wade, W.S., Miller, M.F., Perun, T.J., Holzman, T.F., and Krafft, G.A. (1996). The nanometer-scale structure of amyloid- β visualized by atomic force microscopy. *J. Protein Chem.* 15:193–203.

- Sunde, M., and Blake, C.C.F. (1998). From the globular to the fibrous state: protein structure and structural conversion in amyloid formation. *Q. Rev. Biophys.* 31:1–39.
- Tenidis, K., Waldner, M., Bernhagen, J., Fischle, W., Bergmann, M., Weber, M., Merkle, M.-L., Voelter, W., Brunner, H., and Kapurniotu, A. (2000). Identification of a penta- and hexapeptide of islet amyloid polypeptide (IAPP) with amyloidogenic and cytotoxic properties. *J. Mol. Biol.* 295:1055–1071.
- Tseng, H.-C., Lu, Q., Henderson, E., and Graves, D.J. (1999). Phosphorylated tau can promote tubulin assembly. *Proc. Natl. Acad. Sci. USA.* 96:9503–9508.
- Vivo, M., de Vera, N., Cortes, R., Mengod, G., Camon, L., and Martinez, E. (2001) Polyamines in the basal ganglia of human brain. Influence of aging and degenerative movement disorders. *Neurosci. Lett.* 304:107–111.
- Volles, M.J., Lee, S.-J., Rochet, J.-C., Shtilerman, M.D., Ding, T.T., Kessler, J.C., and Lansbury, P.T., Jr. (2001). Vesicle permeabilization by protofibrillar α -synuclein: implications for the pathogenesis and treatment of Parkinson's disease. *Biochemistry* 40:7812–7819.
- Yang, D.-S., Yip, C.M., Jackson Huang, T.H., Chakrabartty, A., and Fraser, P.E. (1999). Manipulating the amyloid- β aggregation pathway with chemical chaperones. *J. Biol. Chem.* 274:32970–32974.
- Yip, C.M., and McLaurin, J. (2001). Amyloid- β peptide assembly: a critical step in fibrillogenesis and membrane disruption. *Biophys. J.* 80:1359–1371.
- Yip, C.M., Darabie, A.A., and McLaurin, J. (2002). A β 42-peptide assembly on lipid bilayers. *J. Mol. Biol.* 318:97–107.
- Yip, C.M., Elton, E.A., Darabie, A.A., Morrison, M.R., and McLaurin, J. (2001). Cholesterol, a modulator of membrane-associated A β -fibrillogenesis and neurotoxicity. *J. Mol. Biol.* 311:723–734.
- Žerovnik, E. (2002). Amyloid-fibril formation: Proposed mechanisms and relevance to conformational disease. *Eur. J. Biochem.* 269:3362–3371.
- Zhu, M., Li, J., and Fink, A.L. (2003). The association of α -synuclein with membranes affects bilayer structure, stability and fibril formation. *J. Biol. Chem.* 278:40186–40197.
- Zhu, M., Souillac, P.O., Ionescu-Zanetti, C., Carter, S.A., and Fink, A.L. (2002). Surface-catalyzed amyloid fibril formation. *J. Biol. Chem.* 277:50914–50922.

Direct Observation of Amyloid Fibril Growth Monitored by Total Internal Reflection Fluorescence Microscopy

Tadato Ban and Yuji Goto

1. Abstract

Amyloid fibril formation is a phenomenon common to many proteins and peptides associated with numerous conformational diseases. To clarify the mechanism of fibril formation and to create inhibitors, real-time monitoring of fibril growth is essential. This chapter describes a method to visualize amyloid fibril growth in real time at the single fibril level. This approach uses total internal reflection fluorescence microscopy (TIRFM) combined with the binding of thioflavin T, an amyloid-specific fluorescence dye. The method enables an exact analysis of the rate of growth of individual fibrils. One of the advantages of TIRFM is that only amyloid fibrils lying in parallel with the slide glass surface were observed, so that one can obtain the exact length of fibrils. This method is of particular importance for the analysis of rapid fibrillation kinetics, providing unique information crucial for the elucidation of the molecular mechanisms of amyloid fibril formation.

2. Introduction

Amyloid fibril formation is considered to be a nucleation-dependent process in which nonnative precursor proteins slowly associate to form the nuclei (Naiki *et al.*, 1997; Dobson, 2003). This process is followed by an extension reaction, where the nucleus grows by sequential incorporation of more precursor protein molecules. This model has been validated by the observation that fibril-extension kinetics is accelerated by the addition of preformed fibrils, that is, by a seeding effect. However, the mechanism of fibril formation by individual polypeptide chains is not completely understood, and there are several variations of the nucleation-dependent model (Scheibel *et al.*, 2001; Depace and Weissman, 2002; Kad *et al.*, 2003). To address the mechanism of amyloid fibril formation, it is important to observe the process at the single-fibril level. Recently, epifluorescence with a newly introduced fluorescent dye (Inoue *et al.*, 2001; Ban *et al.*, 2003) and atomic force microscopy (AFM) (Goldsbury *et al.*, 1999; Ionescu-Zanetti *et al.*, 1999; Kad *et al.*, 2003; Hoyer *et al.*, 2004) have been utilized for the direct observation of individual amyloid fibrils. Although these techniques are quite useful in providing information on the mode of fibril growth, the need to introduce the fluorescence probe prevents their general application. On the other hand, the strong interaction of amyloid proteins and the mica surface used in AFM measurements resulted in the formation of fibrils morphologically different from the intact amyloid fibrils (Goldsbury *et al.*, 1999; Green *et al.*, 2004).

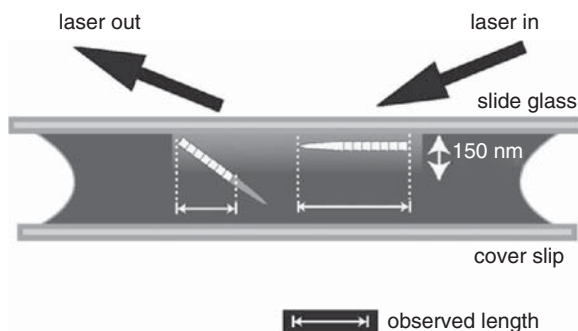


Figure 12.4-1. Schematic representation of amyloid fibrils by total internal reflection fluorescence microscopy. The penetration depth of the evanescent field formed by the total internal reflection of laser light is ~ 150 nm for laser light at 455 nm, so that only amyloid fibrils lying in parallel with the slide glass surface were observed. (Taken and modified from Ban *et al.*, 2003, with permission.)

Thioflavine T (ThT) is known to bind rapidly to amyloid fibrils accompanied by a dramatic increase of fluorescence at around 485 nm, when excited at 455 nm (Naiki *et al.*, 1989; Naiki and Gejyo, 1999). This makes ThT one of the most useful probes to detect the formation of amyloid fibrils. Fluorescence at around 485 nm becomes useful in fluorescence microscopic studies that make use of lasers for the incident beam of excitation. On the other hand, a total internal reflection fluorescence microscope (TIRFM) has been developed to monitor single molecules (Funatsu *et al.*, 1995; Yamasaki *et al.*, 1999; Wazawa *et al.*, 2000) by effectively reducing the background fluorescence under the evanescent field formed on the surface of slide glass. When a laser is incident on an interface between a quartz slide glass (high reflection index) and the aqueous solution (low reflection index) at the critical angle for total internal reflection, the evanescent field is produced beyond the interface in the solution with a penetration depth of about 150 nm. In the analysis of amyloid fibrils, the evanescent field turns out to be very useful for the following reason. To obtain the exact length of fibrils by conventional epifluorescence microscopy, one has to analyze the image by three-dimensional reconstruction because the orientation of fibrils relative to slide glass is not always parallel to the glass surface. In contrast, because the penetration depth of the evanescent field formed by the total internal reflection of laser light is quite shallow (~ 150 nm for laser light at 455 nm), TIRFM selectively monitors long fibrils lying along the slide glass, so that the length of observed fibrils is close to the exact length (Figure 12.4-1). By combining amyloid fibril-specific ThT fluorescence and TIRFM, it has become possible to observe the amyloid fibrils and their formation process without introducing any fluorescence reagent covalently bound to the protein molecule.

3. Experimental Procedures

3.1. Fluorescence Microscopy

The fluorescence microscopic system used to observe individual amyloid fibrils was developed based on an inverted microscope (IX70; Olympus, Tokyo, Japan) as described (Funatsu *et al.*, 1995; Yamasaki *et al.*, 1999; Wazawa *et al.*, 2000). The ThT molecule was excited using an Ar laser (Model 185F02-ADM; Spectra Physics, Mountain View, CA). The 460 nm line of Ar laser was depolarised by passing through a quarter-wave plate. After passing a quartz cubic prism, the laser was incident

on a quartz slide at the incident angle 68° . The gap between the quartz slide and cubic prism was filled with fluorescence-free glycerol. The fluorescence was collected with an oil-immersion microscope objective lens (1.40 NA, 100 X, PlanApo; Olympus, Tokyo, Japan). Fluorescence image was filtered with a bandpass filter (D490/30; Omega Optical, Brattleboro, VT) and was visualized using an image intensifier (Model VS4-1845; Video scope International, Sterling, VA) coupled with a SIT camera (C2400-08; Hamamatsu Photonics, Shizuoka, Japan). Recently, TIRFM microscopy is commercially available from some companies (Model IX71-ARCEVA; Olympus, Tokyo, Japan, and Model TIRF-C1; Nikon, Tokyo, Japan).

3.2. Direct Observation of Amyloid Fibrils

β 2-m amyloid fibrils were prepared as described with recombinant human β 2-m (Naiki *et al.*, 1997; Katou *et al.*, 2002; Kozhukh *et al.*, 2002; Chiba *et al.*, 2003). Seed fibrils (i.e., fragment fibrils) were prepared by sonication of 200- μ l aliquots of a 0.1 mg/ml fibril stock solution using a UltraS homogenizer (VP-60S; TAITEC, Saitama, Japan) at output level 3, 30×1 -second pulses on ice. Seed fibrils were mixed with 25 μ M monomeric β 2-m in polymerization buffer (50 mM Na citrate, pH 2.5, and 100 mM KCl) at 37°C . After 6 hours of incubation, the sample solution was diluted 10-fold with polymerization buffer and 100 μ M ThT was added at the final concentration of 5 μ M. An aliquot (14 μ l) of sample solution was deposited on quartz slide glass, and the fibril image was obtained with TIRFM. All images were recorded on digital videotape and analyzed using Image-pro Plus (Media Cybernetics, Silver spring, MD).

Synthesized medC fragment (NFGSVQFV) and A β (1–40) were purchased from Peptide Institute, Inc. (Osaka, Japan). A β (1–40) amyloid fibrils were prepared from synthetic A β (1–40) (Hasegawa *et al.*, 1999, 2002). To obtain seeds, pre-formed fibrils were fragmented by sonication as described above. The seeds were added at a final concentration of 10 μ g/ml to 50 μ M monomeric A β (1–40) in 50 mM sodium phosphate buffer at pH 7.5 and 100 mM NaCl. After a 3-hour incubation at 37°C in the test tube, the solution was diluted fivefold and ThT was added at a final concentration of 5 μ M. The fibril formation of β 2-m or A β (1–40) on the slide glass was also examined.

3.3. Time-Lapse Observation of Amyloid Fibrils

In the case of β 2-m, seed fibrils were mixed with 50 μ M monomeric β 2-m in polymerization buffer (50 mM Na citrate at pH 2.5 and 100 mM KCl). After ThT was added at 5 μ M, the solution was deposited on quartz slide glass and the growth of individual fibrils was observed every 15 minutes under a microscope at 37°C . For MedC, seed fibrils were prepared by incubating the monomeric peptide at 1 mM in 10 mM Na phosphate buffer (pH 7.0) at 37°C for 24 hours. The fibrils were fragmented by a sonicator as described above. An aliquot (1 μ L) of the seed solution was mixed with 1 mM medC monomer in the same buffer containing 5 μ M ThT, and deposited on quartz slide glass at 37°C for visualization with TIRFM. The images of amyloid fibrils grown under TIRFM recorded on digital video tape were captured on a personal computer, and the lengths of the fibrils were calculated using Image-pro Plus.

4. Results and Discussion

4.1. ThT Observation of β 2-m Amyloid Fibrils

β 2-m, a 99-residue protein with a typical immunoglobulin domain fold (Bjorkman *et al.*, 1987), is the light chain of the major histocompatibility complex class I antigen. However, it is also found as a major component of amyloid fibrils deposited in dialysis-related amyloidosis, a common and

serious complication in patients receiving hemodialysis for more than 10 years (Gejyo *et al.*, 1985; Naiki *et al.*, 1997). Although the exact mechanism of the β 2-m amyloid fibril deposition *in vivo* is still unknown, amyloid fibrils are easily formed *in vitro* by a seed-dependent extension reaction at pH 2.5, in which acid-unfolded monomeric β 2-m is added to seed fibrils taken from patients (Naiki *et al.*, 1997; Katou *et al.*, 2002; Kozhukh *et al.*, 2002).

We first examined the β 2-m amyloid fibrils already extended in test tubes (Figure 12.4-2A and B). TIRFM images indicated the presence of fibrils 1–5 μ m long in the presence of ThT. No such fibrillar structures were found either in the absence of ThT or in the absence of fibrils. This indicated

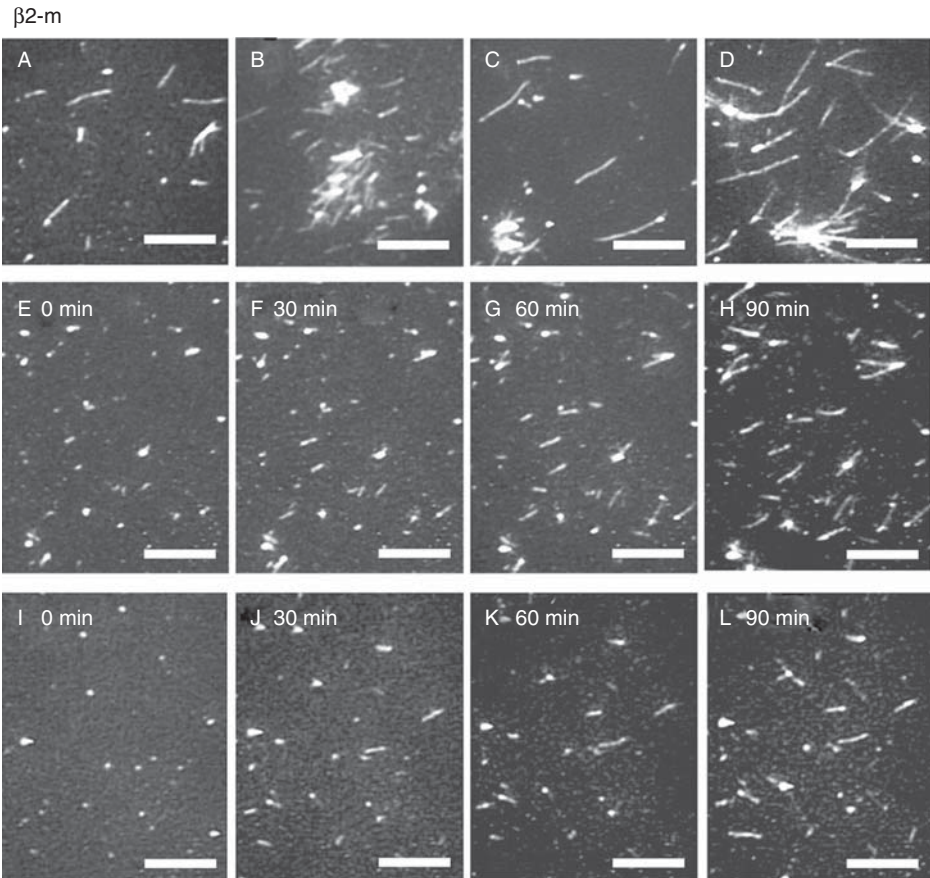


Figure 12.4-2. Images of β 2-m amyloid fibrils observed by ThT fluorescence and TIRFM. (A, B) Amyloid fibrils prepared in a test tube and (C, D) on the slide glass. (E–H) and (I–L): Growth processes of β 2-m amyloid fibrils. In panels (H) and (L), ThT fluorescence at time 0 was indicated to identify the locations of seed fibrils. The scale bars were 10 μ m. (Taken and modified from Ban *et al.*, 2003, with permission.)

that amyloid-specific fluorescence from ThT enables one to visualize the β 2-m amyloid fibrils. The length range is similar to that observed with electron microscopy (EM) (Naiki *et al.*, 1997; Kozhukh *et al.*, 2002) or AFM (Katou *et al.*, 2002). On the other hand, the apparent width of the fibrils observed by fluorescence was larger than the exact size (i.e., about 15 nm from EM) because the observed emission fields extend the dye localisation.

We then determined whether β 2-m amyloid fibrils also formed on the slide glass. Goldsbury *et al.* (1999) reported using synthetic human amylin where amylin fibrils that assembled on a mica surface for AFM measurement exhibited distinct morphological features. The seeds, that is, fragmented fibrils, were mixed with monomeric β 2-m and immediately the solution was deposited on quartz slide glass. As can be seen, the amyloid fibrils extended on the slide glass with an incubation time of 6 hours (Figure 12.4-2C and D) were similar to those prepared in the test tube (Figure 12.4-2A and B).

4.2. Kinetics of Fibril Extension

We monitored the seed-dependent extension reaction (Figure 12.4-2E–L). At time zero, we identified the location of seeds. As we increased the incubation time, we could clearly follow the extension of fibrils: the extension ended at around 2 hours under the conditions used. This fact constitutes direct evidence that the fibril formation by β 2-m is a seed-dependent process, as suggested for other amyloid fibrils (Goldsbury *et al.*, 1999; Inoue *et al.*, 2001; Scheibel *et al.*, 2001; Depace and Weissman, 2002). A majority of extended β 2-m fibrils exhibited unidirectional elongation from the seeds. Moreover, when the fibrils with bidirectional elongation were observed, the superposition of the seeds was suggested. Therefore, we can conclude that the elongation is mostly unidirectional.

Unidirectional fibril formation was first observed using Sup35, a yeast prion determinant, by epifluorescence microscopy (Inoue *et al.*, 2001). However, another group reported the bidirectional elongation of Sup35 based on the observations with EM in conjunction with selective staining by gold particles (Scheibel *et al.*, 2001). Although we cannot exclude the possibility that the interaction with the glass surface was responsible for the unidirectional extension, the unidirectional picture is likely to hold for the fibril formation of β 2-m. The unidirectional elongation was also dominant in the formation of fibrils by medC (see below).

The rate of extension of individual amyloid fibrils was analyzed by plotting the length of fibrils as a function of time (Figure 12.4-3A). For the respective fibrils, the extension reaction could be well fitted to a single exponential curve, consistent with a previous observation of the seed-dependent extension reaction in test tubes (Naiki *et al.*, 1997; Kozhukh *et al.*, 2002; Ohhashi *et al.*, 2002). Importantly, the rates of fibril extension, however, varied significantly depending on the fibrils, although the rate for each fibril remained constant. The initial fibril growth rate showed a wide distribution with a mean value of $47.4 \pm 15.0 \text{ nm min}^{-1}$ (Figure 12.4-3B), which cannot be explained by the statistical distribution of the fibril growth rate. Taking into account the fact that the extension rate for each fibril is constant, the diversity in the rate may be related to the difference in the structure of individual fibrils. Recently, the direct observation of fibril formation by AFM indicated that the fibril-forming region of Sup35 forms a diverse population of fibrils that could be distinguished on the basis of their kinetic properties, including polarity and elongation rate (Depace and Weissman, 2002). Furthermore, another study with NMR (Ippel *et al.*, 2002) indicated that amyloid fibrils formed by the $A\beta(25-35)$ peptide exhibit a heterogeneity in the kinetics of their hydrogen/deuterium exchange behaviour for each amide group. Thus, current data obtained for β 2-m as well as the results discussed for Sup35 and $A\beta$ peptide suggest that heterogeneity of structure is a common characteristic of amyloid fibrils.

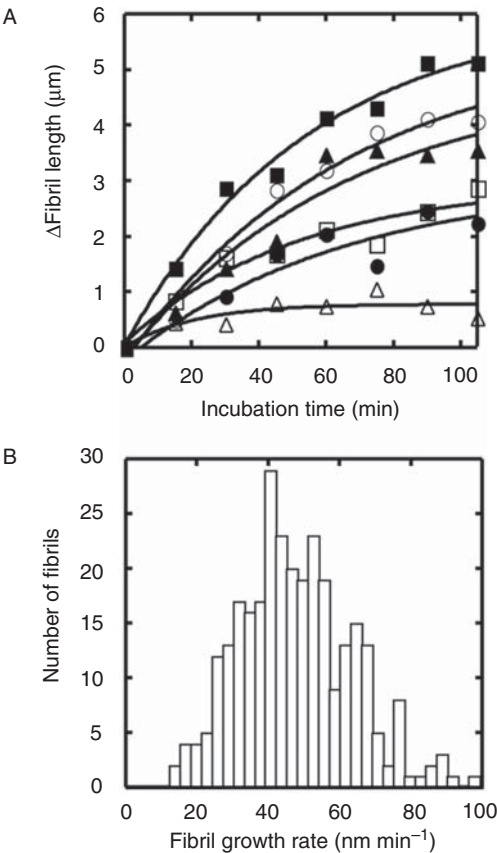


Figure 12.4-3. Kinetics of β 2-m amyloid fibril growth. (A) Fibril length versus incubation time. Lines show the fitting to the pseudo first-order kinetics. (B) Histogram for the distribution of the initial fibril growth rate. (Taken from Ban *et al.*, 2003, with permission.)

4.3. Medin Fragment and A β (1–40)

To confirm the overall applicability of this method, we examined two other amyloid fibrils. One is medC, corresponding to the C-terminal octapeptide of medin (Häggqvist *et al.*, 1999). Medin, a 50-amino acid internal cleavage peptide of lactadherin, is a component of the very common age-related amyloidosis deposited on the aortic wall. It has been shown that the C-terminal 8 amino acid peptide NFGSVQFV from human medin is associated with amyloid fibrils at neutral pH and 37°C (Häggqvist *et al.*, 1999). We first prepared the seed fibrils. In the case of medC, it was difficult to prepare extensively fragmented seeds by sonication. This might be related to the very rigid and sharp morphology of the medC fibrils. The extension reaction was monitored every 5 minutes under microscopic conditions (Figure 12.4-4A–D). We observed the extension of the fibrils, which was mostly unidirectional, as was the case for β 2-m. Analysis of the extension rate also indicated significant heterogeneity of the extension rate (data not shown).

Another example is A β (Figure 12.4-4E–H). The intracerebral accumulation of A β as senile plaques or vascular amyloid is one of the dominant characteristics in the pathogenesis of Alzheimer's

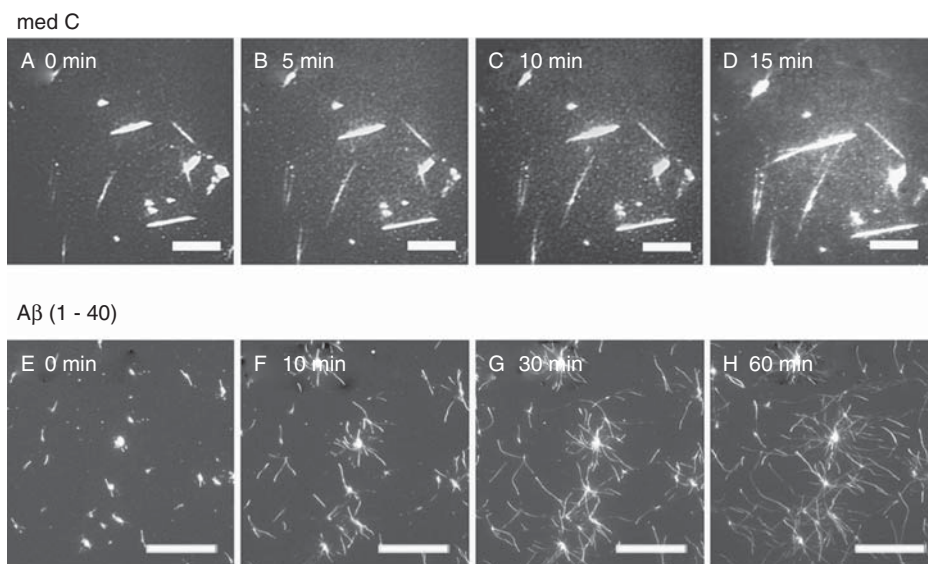


Figure 12.4-4. Applicability of the ThT method to other amyloid fibrils. Real-time monitoring of amyloid fibril growth of medC (A–D) and Aβ(1–40) (E–H). The scale bars were 10 μm. (Taken and modified from Ban *et al.*, 2003, with permission.)

disease, and has been suggested to play a central role, probably as an early event in the amyloid cascade (Hardy and Higgins, 1992; Hardy and Selkoe, 2002; Mattson, 2004). Real-time observation of the growth of individual fibrils following seed-dependent extension was carried out on the surface of glass slides (Figure 12.4-4E–H). At first, the ThT fluorescent spots of the seeds were observed (Figure 12.4-4E). The growth of fibrils occurred concomitantly at many seeds. Although several fibrils often developed from apparently one seed, it is likely that the clustered seeds produced such a radial pattern.

5. Conclusion

By monitoring ThT fluorescence with TIRFM, we showed amyloid fibrils of β2-m can be visualized without requiring covalent fluorescence labeling. Because ThT binding is common to all amyloid fibrils, the present method will have general applicability for the analysis of amyloid fibrils. One of the advantages of TIRFM is that only amyloid fibrils lying in parallel with the slide glass surface were observed, so that we can obtain the exact length of fibrils. Consequently, the method will be particularly important for following the rapid kinetics of fibril formation, which is paramount to elucidating the mechanism of amyloid fibril formation and being less accessible by other approaches.

6. Abbreviations

A β	Amyloid β peptide
AFM	Atomic force microscopy
ThT	Thioflavine T
TIRFM	Total internal reflection fluorescence microscope

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References

- Ban, T., Hamada, D., Hasegawa, H., Naiki, H., and Goto, Y. (2003). Direct observation of amyloid fibril growth monitored by thioflavin T fluorescence. *J. Biol. Chem.* 278:16462–16465.
- Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L., and Willy, D.C. (1987). Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* 329:506–512.
- Chiba, T., Hagiwara, Y., Higashimura, T., Hasegawa, K., Naiki, H., and Goto, Y. (2003). Amyloid fibril formation in the context of full-length protein: effects of proline mutations on the amyloid fibril formation of β_2 -microglobulin. *J. Biol. Chem.* 278:47016–47024.
- Depace, A.H., and Weissman, J. S. (2002). Origins and kinetic consequences of diversity in Sup35 yeast prion fibrils. *Nat. Struct. Biol.* 9:389–396.
- Dobson, C.M. (2003). Protein folding and misfolding. *Nature* 426:884–889.
- Funatsu, T., Harada, Y., Tokunaga, M., Saito, K., and Yanagida, T. (1995). Imaging of single fluorescent molecules and individual ATP turnovers by single myosin molecules in aqueous solution. *Nature* 374:555–559.
- Gejyo, F., Yamada, T., Odani, S., Nakagawa, Y., Arakawa, M., Kunitomo, T., Kataoka, H., Suzuki, M., Nirasawa, Y., Shirohama, T., Cohen, A.S., and Schmid, K. (1985). A new form of amyloid protein associated with chronic hemodialysis was identified as β_2 -microglobulin. *Biochem. Biophys. Res. Commun.* 129:701–706.
- Goldsbury, C., Kistler, J., Aebi, U., Arvinte, T., and Cooper, G.J. (1999). Watching amyloid fibrils grow by time-lapse atomic force microscopy. *J. Mol. Biol.* 285:33–39.
- Green, J.D., Goldsbury, C., Kistler, J., Cooper, G.J.S., and Aebi, U. (2004). Human amylin oligomer growth and fibril elongation define two distinct phases in amyloid formation. *J. Biol. Chem.* 279:12206–12212.
- Hardy, J.A., and Higgins, G.A. (1992). Alzheimer's disease: the amyloid cascade hypothesis. *Science* 256:184–185.
- Hardy, J., and Selkoe, D.J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297:353–356.
- Hasegawa, K., Yamaguchi, I., Omata, S., Gejyo, F., and Naiki, H. (1999). Interaction between A β (1–42) and A β (1–40) in Alzheimer's β -amyloid fibril formation *in vitro*. *Biochemistry* 38:15514–15521.
- Hasegawa, K., Ono, K., Yamada, M., and Naiki, H. (2002). Kinetic modeling and determination of reaction constants of Alzheimer's β -amyloid fibril extension and dissociation using surface plasmon resonance. *Biochemistry* 41: 13489–13498.
- Haggqvist, B., Näslund, J., Sletten, K., Westermark, G.T., Mucchiano, G., Tjernberg, L.O., Nordstedt, C., Engström, U., and Westermark, P. (1999). Medin: an integral fragment of aortic smooth muscle cell-produced lactadherin forms the most common human amyloid. *Proc. Natl. Acad. Sci. USA* 96:8669–8674.
- Hoyer, W., Cherny, D., Subramaniam, V., and Jovin T.M. (2004). Rapid self-assembly of α -synuclein observed by *in situ* atomic force microscopy. *J. Mol. Biol.* 340:127–139.
- Inoue, Y., Kishimoto, A., Hirao, J., Yoshida, M., and Taguchi, H. (2001). Strong growth polarity of yeast prion fiber revealed by single fiber imaging. *J. Biol. Chem.* 276:35227–35230.

- Ionescu-Zanetti, C., Khurana, R., Gillespie, J.R., Petrick, J.S., Trabachino, L.C., Minert, L.J., Carter, S.A., and Fink, A.L. (1999). Monitoring the assembly of Ig light-chain amyloid fibrils by atomic force microscopy. *Proc. Natl. Acad. Sci. USA* 96:13175–13179.
- Ippel, J.H., Olofsson, A., Schleucher, J.S., Lundgren, E., and Wijmenga, S.S. (2002). Probing solvent accessibility of amyloid fibrils by solution NMR spectroscopy. *Proc. Natl. Acad. Sci. USA* 99:8648–8653.
- Kad, N.M., Myers, S.L., Smith, D.P., Smith, D.A., Radford, S.E., and Thomson, N.H. (2003). Hierarchical assembly of β_2 -microglobulin amyloid *in vitro* revealed by atomic force microscopy. *J. Mol. Biol.* 330:785–797.
- Katou, H., Kanno, T., Hoshino, M., Hagihara, Y., Tanaka, H., Kawai, T., Hasegawa, K., Naiki, H., and Goto, Y. (2002). The role of disulfide bond in the amyloidogenic state of β_2 -microglobulin studied by heteronuclear NMR. *Protein Sci.* 11:2219–2229.
- Kozhukh, G.V., Hagihara, Y., Kawakami, T., Hasegawa, K., Naiki, H., and Goto, Y. (2002). Investigation of a peptide responsible for amyloid fibril formation of β_2 -microglobulin by *Acromobacter* protease I. *J. Biol. Chem.* 277: 1310–1315.
- Mattson M.P. (2004). Pathways towards and away from Alzheimer's disease. *Nature* 430:631–639.
- Naili, H., and Gejyo, F. (1999). Kinetic analysis of amyloid fibril formation. *Methods Enzymol.* 309:305–318.
- Naiki, H., Higuchi, K., Hosokawa, M., and Takeda, T. (1989). Fluorometric determination of amyloid fibrils *in vitro* using the fluorescent dye, thioflavin T. *Anal. Biochem.* 177:244–249.
- Naiki, H., Hashimoto, N., Suzuki, S., Kimura, H., Nakakuki, K., and Gejyo, F. (1997). Establishment of a kinetic model of dialysis-related amyloid fibril extension *in vitro*. *Amyloid* 4:223–232.
- Ohhashi, Y., Hagihara, Y., Kozhukh, G., Hoshino, M., Hasegawa, K., Yamaguchi, I., Naiki, H., and Goto, Y. (2002). The intrachain disulfide bond of β_2 -microglobulin is not essential for the immunoglobulin fold at neutral pH, but is essential for amyloid fibril formation at acidic pH. *J. Biochem.* 131:45–52.
- Scheibel, T., Kowal, A.S., Bloom, J.D., and Lindquist, S.L. (2001). Bidirectional amyloid fiber growth for a yeast prion determinant. *Curr. Biol.* 11:366–369.
- Wazawa, T., Ishii, Y., Funatsu, T., and Yanagida, T. (2000). Spectral fluctuation of a single fluorophore conjugated to a protein molecule. *Biophys. J.* 78:1561–1569.
- Yamasaki, R., Hoshino, M., Wazawa, T., Ishii, Y., Yanagida, T., Kawata, Y., Higurashi, T., Sakai, K., Nagai, J., and Goto, Y. (1999). Single molecular observation of the interaction of GroEL with substrate proteins. *J. Mol. Biol.* 292: 965–972.

Animal and Cell Models of Human Neurodegenerative Disorders

Drosophila* and *C. elegans

Models of Human Age-Associated Neurodegenerative Diseases

Julide Bilen and Nancy M. Bonini

1. Abstract

Defining specific mutations in familial human neurodegenerative diseases has allowed researchers to make animal models of the diseases through directed genetic approaches. These studies help address the molecular mechanisms of disease, and provide the foundation toward therapeutics. Modeling human neurodegenerative diseases in invertebrates has revolutionized the field in such a way that both reverse and forward genetic approaches are leading to the discovery of new players in neurodegeneration. This review focuses on fruit fly and nematode models of human neurodegenerative diseases, with emphasis on how these models have provided new insights into aspects of human disease.

2. Introduction

Dysfunction and/or progressive degeneration of subsets of neurons in the brain leads to several human neurodegenerative diseases associated with various devastating clinical symptoms such as ataxia, tremor and movement disorders, or cognitive and memory loss. These diseases include Parkinson's disease (PD), Alzheimer's disease (AD), frontotemporal dementia with parkinsonism (FTDP), triple nucleotide expansion diseases, and many others (Ross *et al.*, 1997, 1999; Lang and Lozano, 1998; Price *et al.*, 1998; Perutz, 1999; Fortini and Bonini, 2000; Zoghbi and Orr, 2000; Lee *et al.*, 2001; Hardy and Selkoe, 2002; Bonini and Fortini, 2003; Driscoll and Gerstbrein, 2003). Among those, triple repeat expansion diseases are classified into two groups: type I diseases, in which the repeat expansion occurs within the open reading frame of the gene, often encoding an expanded polyglutamine domain such as in Huntington's disease (HD), several Spinocerebellar Ataxias (SCAs) and Spinobulbar Muscular Atrophy (SBMA) disease; and type II diseases, in which the triplet repeat expansion occurs within noncoding sequence, as in Fragile X syndrome (Cummings and Zoghbi, 2000). Although most disease is sporadic, familial forms of these diseases have also been found, which provide a handle on a gene function that is altered. Some of these diseases are dominant, others recessive, and some influenced by environmental circumstances. A major pathological characteristic shared by most neurodegenerative diseases is the formation of insoluble protein accumulations, suggesting potential common defects in protein folding and degradation.

Although, classically, researchers have modeled human disease in cell lines or the mouse, the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* offer many advantages

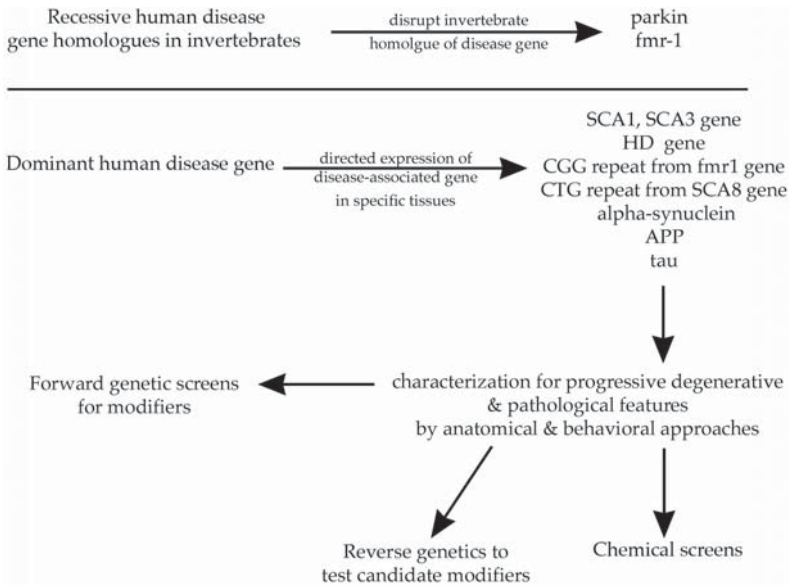


Figure 13.1-1. Genetic experimental approaches to human neurodegenerative disease modeling in invertebrate systems. General approach to modeling human diseases in invertebrates includes both knock-out of gene function for select diseases, as well as a directed expression approach for dominant gain-of-function diseases. With such models in hand, one can then take a number of approaches, including studying the phenotype to learn of disease mechanisms, as well as genetic and chemical approaches for modifiers

for studying molecular mechanisms of human neurodegenerative diseases. Among the great benefits of the fly and nematode model systems are the efficacy of forward genetic screens, which can reveal novel components of the biological process of interest, without requiring prior knowledge of mechanism so in an unbiased way (Figure 13.1-1). In addition, these organisms have the benefit of allowing drug and chemical screening, due to a minimal blood–brain barrier. Both flies and nematodes have shorter life spans, large number of progeny, known genomic sequence, and well-established molecular genetic techniques for the study of gene function. *Drosophila* and *C. elegans* also have a high degree of conservation to humans in fundamental biological pathways revealed by genetic studies and genome sequence analysis (Bargmann, 1998; Rubin *et al.*, 2000). Here, we review fruit fly and nematode models of polyglutamine disease, noncoding triplet repeat diseases, PD, AD, and FTDP, and the genetic and pharmacological modifiers of neurodegeneration identified via either forward or reverse approaches with the use of these models.

3. Modeling Human Polyglutamine Diseases

3.1. Human Polyglutamine Diseases

Discovery of a CAG trinucleotide expansion within the open reading frame of the androgen receptor gene as the molecular basis of SBMA (also known as Kennedy's disease) in the early 1990s by LaSpada *et al.* (1991) turned out to herald identification of a molecular basis now known to underlie

at least nine dominantly inherited human neurodegenerative diseases. These diseases include HD, several Spinocerebellar Ataxias (SCA1, 2, 3, 6, 7, and 17), and Dentatorubral-Pallidoluysian Atrophy (DRPLA) (Ross *et al.*, 1997, 1999; Perutz, 1999; Fortini and Bonini, 2000; Zoghbi and Orr, 2000; Bonini and Fortini, 2003; Driscoll and Gerstbrein, 2003), which share a common “CAG repeat expansion” in otherwise unrelated genes. The imminent question raised was how a CAG repeat expansion causes progressive neurodegeneration in subsets of neurons in the brain. Perutz proposed that the extended polyglutamine stretch encoded by the CAG repeat structurally alters the protein via formation of a β -pleated sheet structure through hydrogen bonding in an intra- and intermolecular manner (Perutz *et al.*, 1994). An alternative hypothesis is that the enzyme transglutaminase might covalently crosslink the disease protein (Green, 1993). These structural alteration(s) presumably trigger subsequent events that are toxic to neurons. Mouse models of SCA1-Q82, truncated SCA3-Q79, and HD exon-1-Q140 and a polyglutamine expansion in HPRT (an unrelated protein not found mutated in a human polyglutamine disease) showed neurological dysfunction and degeneration of neurons, indicating that the polyQ domain per se has a toxic gain-of-function effect, with host protein context among the factors conferring cellular specificity (Burrigh *et al.*, 1995; Ikeda *et al.*, 1996; Mangiarini *et al.*, 1996; Ordway *et al.*, 1997). Histopathological analysis of patient tissues shows that, indeed, the disease protein accumulates in inclusions, which are typically nuclear and immunostain with ubiquitin, suggesting a role for ubiquitin-dependent proteolytic processes in polyglutamine-mediated neurodegeneration (Cummings *et al.*, 1998). Transfection of the pathogenic forms of the disease gene into cell lines indicated that protein inclusions coimmunolabel for subunits of the proteasome and molecular chaperone components of the stress response, suggesting problems with protein misfolding and clearance of the pathogenic protein (Cummings *et al.*, 1998; Chai *et al.*, 1999).

3.2. Modeling Polyglutamine Diseases in *Drosophila melanogaster*

Machado Joseph disease, also known as spinocerebellar ataxia type 3 (MJD/SCA3), was modeled in the fly using the C-terminal portion of human SCA3 with either a normal-length polyglutamine repeat of Q27 (SCA3tr-Q27), or a disease-associated expanded polyglutamine repeat of Q78 (SCA3tr-Q78) (Warrick *et al.*, 1998). This truncated pathogenic protein had previously been shown to cause cerebellar degeneration in transgenic mice (Ikeda *et al.*, 1996). Gene expression was directed using the widely employed GAL4/UAS yeast expression system that was adapted for use in flies to confer conditional, tissue-specific gene expression (Brand and Perrimon, 1993). Directed expression of the pathogenic human disease protein SCA3tr-Q78 in the eye via a glass multiple reporter-gal4 (*gmr-gal4*) driver line causes expression level-dependent external and internal retinal degeneration (**Figure 13.1-2b and c; see color insert**). In contrast, the protein bearing a normal length polyQ repeat SCA3tr-Q27 has no effect. The neurodegeneration seen in the fly remarkably reflects fundamental aspects of the human disease in being late onset and progressive. Toxicity of the protein is not only limited to the retina, but also occurs when targeted to the nervous system using a pan neuronal driver *elav-gal4*. Strikingly, SCA3tr-Q78 also accumulates in nuclear inclusions (**Figure 13.1-2e and f; see color insert**), in contrast to the nondisease control protein, SCA3tr-Q27, which remains diffuse. These studies indicated that fundamental aspects of human polyglutamine diseases can be recapitulated in the fly.

Subsequent modeling of HD in the fly eye using an amino terminal fragment of the Huntingtin protein with 2, 75, or 120 polyQ repeats revealed similar adult-onset, progressive neuronal degeneration for the pathogenic Q75 and Q120 proteins, but not the control Q2 protein (Jackson *et al.*, 1998). Degeneration is faster for the Q120 protein than Q75, revealing a repeat length-dependent degree of toxicity as seen in human disease. The pathogenic protein localizes to the nucleus, although does not form obvious nuclear inclusions over the time course of the fly experiments. A subsequent model

using full-length SCA1 protein with 82Qs also showed progressive neurodegeneration (Fernandez-Funez *et al.*, 2000). In this case, even the SCA1 protein with a control length polyQ repeat (SCA1-Q30) causes a rough eye phenotype, suggesting that toxicity of the SCA1 protein depends not only on polyQ repeat length but also on the expression level of disease protein.

More recently, modeling of SBMA in flies revealed the striking finding that neurodegeneration induced by a pathogenic form of the human androgen receptor (hAR-Q52) is ligand dependent, with androgen binding inducing nuclear translocation and subsequent cellular degeneration. The important event appears to be translocation to the nucleus, because antagonists that also cause nuclear entry of the protein, but fail to activate androgen-dependent gene expression, also cause degeneration (Takeyama *et al.*, 2002). The importance of nuclear translocation in SBMA fly models has been confirmed in transgenic mouse models, where females (who have low androgen) and animals treated with agents to reduce androgen levels display abrogated neurodegeneration (Katsuno *et al.*, 2002).

Pallanck and colleagues (Satterfield *et al.*, 2002) investigated the normal cellular function of the *Drosophila* homologue of human SCA2. They found that both reducing as well as increasing the level of the *Drosophila* gene causes degeneration, presumably by dysregulation of actin filament formation. This suggests that a dose-dependent regulation of cytoskeleton structure may be important for cell survival. Interestingly, Gunawardena *et al.* (2003) found that RNAi-induced loss of function of the *Drosophila* homologue of Huntingtin leads to axonal transport defects and degeneration in the eye. Both studies suggest that the normal functions of these proteins are required for neuronal maintenance.

Even pure polyQ repeats, not in the context of a human disease protein, are highly toxic in flies, inducing severe degeneration (Kazemi-Esfarjani and Benzer, 2000; Marsh *et al.*, 2000). Moreover, Marsh *et al.* (2000) showed that the degenerative activity of an expanded polyglutamine domain (Q108) becomes mitigated with protein context.

3.3. Lessons from Fly Models: Suppressors and Enhancers of PolyQ Toxicity

3.3.1. Chaperones as Suppressors of PolyQ Toxicity

Ubiquitinated nuclear inclusions sequester heat-shock protein 40 (hsp40) and proteasomal subunits in cell line transfection assays, implying that pathogenic polyglutamine protein is recognized as a misfolded protein and suggesting functional roles for chaperones and proteolytic pathways in disease pathology (Cummings *et al.*, 1998). Molecular chaperones, also known as heat-shock proteins, are involved in folding of proteins and/or preventing aggregation of misfolded proteins, presumably by facilitating refolding of the protein or degradation via the ubiquitin-dependent proteolytic system (Parsell *et al.*, 1993; Bukau and Horwich, 1998; Mathew and Morimoto, 1998). Further, coexpression of heat-shock proteins decreases the polyQ aggregation in cell culture assays (Jana and Nukina, 2003).

A role for chaperones in polyQ toxicity *in vivo* was first tested with directed expression of Hsp70. Upregulation of Hsp70 suppresses degeneration induced by SCA3tr-Q78 (**Figure 13.1-2c; see color insert**) and HD exon1-Q120, while expression of a dominant negative form of the constitutively expressed hsc70 enhances polyQ toxicity (Warrick *et al.*, 1999; Chan *et al.*, 2000). It was subsequently found that two cochaperones, Hsp40 and the *Drosophila* homologue of tetratricopeptide repeat protein 2, are also suppressors of polyQ toxicity (Chan *et al.*, 2000; Fernandez-Funez *et al.*, 2000; Kazemi-Esfarjani and Benzer, 2000). Hsp40 and heat-shock response factor hsr- ω were identified in a genome-wide screen as modifiers of SCA1-induced pathology (Fernandez-Funez *et al.*, 2000). These *in vivo* data emphasize that the stress response may be a common pathway by which pathogenic polyQ

protein toxicity can be modulated. Coexpression of molecular chaperones typically suppresses without an obvious change in the aggregation of the protein, except for SCA1-Q82, where Hsp40 reduces protein accumulation. Chan *et al.* (2000) also showed that chaperones increase the solubility of polyQ inclusions by biochemical assays.

Components of the ubiquitin proteasomal system (UPS) have also been identified as modifiers of polyQ diseases (Chan *et al.*, 2000; Fernandez-Funez *et al.*, 2000). Moreover, loss of function of ubiquitin enhances SCA1 toxicity indicating that ubiquitination may normally ameliorate the toxicity of the pathogenic protein. In the same screen, loss of function of two different ubiquitin conjugases, UbcD1 and dUbc-E2H, were also found to be enhancers. However, whether the disease proteins are direct targets of ubiquitination and/or modulation of other target(s) is what leads to modulation of toxicity remains to be answered.

Steffan *et al.* (2004) showed that the Huntingtin exon1 protein (Httexon1-Q97) is directly modified in cell culture via either ubiquitin or ubiquitin-like proteins (SUMO) on lysine residues. They found that a 50% reduction of SUMO gene activity decreases the extent of Httexon1-Q97-induced photoreceptor neurodegeneration. In addition, mutations in lysine residues that should be SUMOylated *in vivo* mitigate the toxicity of the protein, suggesting that SUMOylation normally exacerbates the toxicity of pathogenic Htt. On the contrary, a mutant form of the SUMO-1-activating enzyme Uba-2 enhances ARtr-Q112-induced neurodegeneration, suggesting that SUMOylation may normally suppress AR-induced toxicity (Chan *et al.*, 2002). Opposite effects of SUMOylation on ARtr-Q112 and Httexon1-Q97 pathogenicity may be due to distinct mechanism(s) of each disease. It will be interesting to know whether SUMOylation affects the fly models bearing full-length disease proteins.

3.3.2. Transcriptional Activity Modulates PolyQ Toxicity

Modulation of transcriptional activity affects polyQ toxicity, and may be affected in disease pathology. The polyQ domain of the Httexon1 interacts with the acetyltransferase domains of CREB-binding protein (CBP) and p300/CBP-associated factor (P/CAF) (Steffan *et al.*, 2001). This interaction was shown to inhibit the acetyltransferase activity of the proteins, indicating that compromised acetylation of chromatin structure may occur in disease. Indeed, the reduction in acetylation can be mitigated via inhibiting the activity of counteracting histone deacetylases (HDAC). Thus, feeding flies HDAC inhibitors (butyrate and SAHA) or reducing the levels of histone acetylases genetically mitigates degeneration induced by Httexon1 in neurons.

Several transcriptional cofactors (Sin3A, Rpd3, dCtBP, dSir2, Pap/Trap, and Tara) have been identified as enhancers of SCA1-induced neurodegeneration in an overexpression modifier screen (Fernandez-Funez *et al.*, 2000). Given that modulation of histone acetylation can either enhance or mitigate degeneration depending upon the disease protein, the effect of such modifiers may depend upon the particular disease protein affected and the cellular balance of proteins involved in the modification pathway in that pathogenic situation.

3.3.3. Pathogenic PolyQ Protein Causes Axonal Transport Defects

Several studies (Gunawardena *et al.*, 2003; Szebenyi *et al.*, 2003; Lee *et al.*, 2004) have raised the hypothesis that pathogenic polyQ protein may induce axonal transport defects in neurons that subsequently leads to neuronal dysfunction and degeneration. Axonal blockages can be visualized by accumulation of polyQ aggregates in axons and dendrites, as well as by swollen axonal morphology. Overexpression of Httexon1-Q93, SCA3tr-Q78, and Q127 in neurons is associated with lethargic movement of larvae and results in excessive TUNEL staining indicative of apoptotic cell death (Gunawardena *et al.*, 2003). PolyQ aggregates may also deplete soluble pools of motor proteins

(dynein and kinesin subunits), which may also contribute to the observed axonal transport defects. Interestingly, axonal transport defects were observed when polyQ protein was directed to the cytoplasm, but not when the pathogenic protein was targeted to the nucleus, suggesting that there are at least two distinct mechanisms of neurodegeneration: one defined by cytoplasmic protein, and the other by nuclear localized protein. Lee *et al.* (2004) also reported reduced depolarization of photoreceptor neurons and elimination of synaptic transmission as a result of directed expression of Htt-128Q, but not Htt-Q0, in the eye. Indeed, mutation of proteins associated with motors can cause human motor neuron degeneration disease (Puls *et al.*, 2003), emphasizing the critical importance of axonal transport in neuronal survival and function.

3.3.4. Additional Modifiers of PolyQ Pathogenicity

A number of RNA binding proteins have been identified as modifiers of SCA1-Q82 toxicity, including overexpression of *mushroom body expressed (mub)* (Fernandez-Funez *et al.*, 2000). Mub has a KH RNA binding domain, and may stabilize specific mRNAs (Grams and Korge, 1998). Whether it suppresses SCA1 neurodegeneration via a specific RNA target or structural interaction between the Mub protein and SCA1 disease protein has not been tested. Moreover, the SCA1 protein itself may have a role as an RNA binding protein (Yue *et al.*, 2001).

A potential role of oxidative stress pathways in disease mechanisms was revealed by identification of glutathione-S transferase (GST) as an overexpression suppressor of SCA1 toxicity (Fernandez-Funez *et al.*, 2000). It will be interesting to know whether GST has such a protective effect for other neurodegenerative diseases, and whether the target is general health of the cell or a specific aspect of the disease pathway.

Other modifiers include the *ter94* gene, which encodes the fly homologue of VCP/CDC48, a member of the AAA+ class of ATPase proteins (Higashiyama *et al.*, 2002). This protein may feed into cell degeneration pathways, as overexpression of *ter94* causes a phenotype with some similarity to polyQ degeneration. Human myeloid leukemia factor 1 (dMLF) has also been found as a suppressor of polyglutamine toxicity (Kazemi-Esfarjani and Benzer, 2002). dMLF colocalizes to polyQ inclusions without changing the accumulation of the polyQ protein, such that the mechanism of suppression is not clear.

The accumulation of the SCA1 disease protein ataxin-1 is strikingly affected by protein phosphorylation, which affects its toxicity. Emamian *et al.* (2003) and Chen *et al.* (2003) revealed that ataxin-1 is phosphorylated by Akt kinase at serine 776, and that mutation of this amino acid to prevent phosphorylation (S776A) prevents aggregate formation in cell culture assays. Zoghbi and colleagues (Chen *et al.*, 2003) identified 14-3-3 as a protein that interacts with the phosphorylated form of SCA1-Q82, but not with the version incapable of being phosphorylated. Overexpression of either 14-3-3 or Akt kinase enhances SCA1-induced neurodegeneration in the fly by increasing the stability of the pathogenic disease protein. These data indicate that modulation of various types of pathways that ultimately affect the accumulation of the pathogenic disease protein may be a very effective approach toward mitigating degeneration. Indeed, small peptides designed to prevent aggregate interactions of polyQ protein have also been shown to be effective suppressors (Kazantsev *et al.*, 2002).

Rubinsztein and colleagues (Ravikumar *et al.*, 2002; Webb *et al.*, 2003) previously found that rapamycin, a stimulator of the autophagy-lysosome pathway, enhances the clearance of polyQ aggregates and PD-associated α -synuclein aggregates in cell culture. In a more recent study, they found that the mammalian target of rapamycin (mTOR) appears to be sequestered in aggregates in cell culture, in a mouse HD model and in the HD patient brain, and the activity of mTOR is reduced in cell culture disease models (Ravikumar *et al.*, 2004). Inhibition of mTOR activity by rapamycin

suppresses photoreceptor degeneration induced by HDexon1-Q74 in the fly and mitigates behavioral performance of the HD mouse model by inducing autophagy and subsequent clearance of the toxic protein via lysosomes.

3.4. *C. elegans* Models of Polyglutamine Disease

Three different nematode models of polyQ disease elegantly use the transparency of this animal, its well-defined neuronal lineage, and the easy use of RNA interference to address aspects of polyQ pathogenicity. Parker *et al.* (2001) expressed the first 57 amino acids of the Huntingtin disease protein with a 19, 88, or 128 polyQ domain fused to GFP, in the touch receptor neurons. The extent of loss of touch sensitivity correlated with the length of the polyQ repeat, while control proteins showed a constant basal level of age-dependent loss of touch sensitivity. They also noted faint perinuclear and nuclear accumulations of the polyQ protein. In this model, directed expression of pathogenic protein does not cause cell death, but shows axonal morphology defects. Faber *et al.* (1999) directed the expression of Htt-Q150 to sensory neurons and found progressive neuronal dysfunction assayed by dye-filling ability of the neurons and nose-touch response assay, although without obvious signs of loss of these neurons. Morley *et al.* (2002) generated GFP linked to various lengths of a pure polyQ domain, finding a threshold polyQ repeat length for aggregate formation. Only the protein with polyQ repeats of 40 or higher forms aggregates in an age-dependent manner, with the extent of motility defects strongly correlating with the polyQ length and aggregate accumulation.

3.5. Lessons from Nematode Models: Suppressors and Enhancers

One of the unresolved issues in the neurodegenerative disease field is the mechanism of neuronal loss, whether death occurs through known cell death pathways or through novel pathways. In the nematode models, expression of toxic polyQ protein does not cause cell death as defined by current apoptotic genes. However, combining toxic polyQ protein with other toxic proteins has been shown to cause progressive cell death of sensory neurons (Faber *et al.*, 1999). This progressive cell death is blocked by mutation of *ced-3*, the major cysteine protease involved in the programmed cell death/apoptotic pathway; however, because two toxic proteins are being expressed, it is hard to know which toxic protein sensitizes and which causes *ced-3* suppressible cell death. In fact, morphological analysis of the neurons in the other HD worm model indicates that the cells do not show the characteristics of apoptosis, such as acridine orange staining (indicative of fragmentation of nuclear DNA), membrane blebbing, and cell shrinkage (Parker *et al.*, 2001).

Morimoto and colleagues (Morley *et al.*, 2002) expressed a pure polyQ domain of Q82 in the background of the *age-1* mutant (the homologue of the human phosphoinositol 3 kinase gene) to test the age dependency of polyQ-induced cell dysfunction. *age-1* mutant nematodes live longer than wild-type animals. Interestingly, both polyQ onset of aggregation and toxicity are delayed in the *age-1* background. However, it remains to be resolved whether extended life span per se slows down the aggregation process or Age-1 has a specific function on polyQ accumulation and toxicity. In a subsequent study, Hsu *et al.* (2003) investigated the roles of two other age-related genes, *heat-shock transcription factor* (*hsf*) and *daf-16*, in polyQ toxicity. They found that reducing the activity of HSF or DAF-16 enhances polyQ aggregation and decreases the nematode life span. These genes regulate the transcription of small heat-shock proteins, which normally delay aggregate formation and increase the life span, suggesting that the mechanism of suppression of polyQ toxicity by genes involved in life span happens through modulation of chaperone activity.

In a modifier screen of Htt-Q150 neuronal dysfunction, a novel nuclear protein, called polyQ enhancer protein (PQE-1), was identified (Faber *et al.*, 2002). Loss of function of *pqe-1* specifically

enhances the loss of Htt-Q150-expressing neurons, but has no effect on other types of degeneration, such as *deg-1* associated loss of touch neurons. Overexpression of *pqe-1* also protects against polyQ-induced neurodegeneration through its glutamine/proline-rich domain, which bears similarity to prion domains.

A genome-wide RNAi screen was performed to reveal modifiers of aggregate formation in the nematode, which emphasized the importance of protein homeostasis in polyQ pathology (Nollen *et al.*, 2004). In this screen, Nollen *et al.* (2004) directed a fluorescently tagged polyQ run (YFP-Q35) to the body wall muscles and looked for enhanced aggregate formation in animals fed bacteria bearing RNAi constructs of 16,757 of the *C. elegans* genes. They identified 186 modifiers that normally function to reduce the aggregate formation of the polyQ, and categorized them into five major groups: RNA synthesis and processing, protein synthesis, protein folding, protein transport, protein degradation, and novel modifiers. It will be interesting to know what extent each modifier group contributes to toxicity of a pathogenic human disease protein in *C. elegans*.

4. Modeling Noncoding Trinucleotide Repeat Diseases

4.1. Noncoding Trinucleotide Repeat Diseases

In the type II trinucleotide diseases, expansion of CGG, CTG, CAG, GCC, and GAA occurs within the 5' or 3' untranslated region (UTR) or in an intron of the respective genes, presumably leading to either loss of function or toxic gain of function, or both, of the disease-associated mRNA (Cummings and Zoghbi, 2000). The repeat range for disease onset varies significantly compared to polyQ diseases. Type II diseases include Fragile X syndrome (CGG expansion in 5' UTR of FMR1), Fragile XE syndrome (GCC expansion in 5' UTR of FMR2), Friedreich's ataxia (GAA expansion in the intron of the *frataxin* gene), myotonic dystrophy type I (CTG expansion in 3' UTR of myotonic dystrophy protein kinase (DMPK) gene), spinocerebellar ataxia type 8 (CTG expansion in the 3' UTR of noncoding SCA8 gene), and SCA12 (CAG expansion in the 5' UTR of a gene encoding one of the regulatory subunit of protein phosphatase 2A, among others). Fragile X syndrome and SCA8 have been modeled in flies.

4.2. Modeling Fragile X in the Fly

Fragile X syndrome is the most commonly inherited mental retardation disorder, with a frequency of 1/4000 (Gao, 2002). It is typically linked to a CGG repeat expansion of more than 230 in the 5' untranslated region of the fragile X mental retardation 1 (*fmr1*) gene. Expansion causes hypermethylation of CpG islands within the *fmr1* promoter, resulting in silencing of the *fmr1* gene transcription, such that loss of function of *fmr1* has a causal role in mental retardation. Two complementary approaches taken in the fly revealed insightful information about the mechanism of disease. First, several groups have created null alleles of the single *Drosophila* homologue of the human FMR1 gene (*dfmr1*), and characterized the behavioral and morphological defects that occur (Zhang *et al.*, 2001; Dockendorff *et al.*, 2002; Morales *et al.*, 2002). They found that homozygous loss of function of *dfmr* causes strong eclosion and circadian rhythm defects. Dockendorff *et al.* (2002) also reported that homozygous *dfmr* loss results in an inconsistent pattern of locomotor activity and reduced courtship behavior without changes in other functions such as phototaxis, chemotaxis, or lifespan. Morales *et al.* (2002) showed that *dfmr* mutant flies have abnormalities in neurite extension, axon guidance, and branching. Both studies suggest that abnormalities in the processes of neurons may account for the behavioral phenotypes observed in the fly. Interestingly, a previous study done by Zhang *et al.*

(2001) revealed that mutation of *futsch*, encoding the *Drosophila* homologue of human microtubule-associated protein MAP1B, mimics the synaptic growth phenotype of *dfmr* null flies. Strikingly, double mutants of *futsch* and *dfmr* have normal synaptic growth and function indicating that *futsch* mRNA may be a major target of FMR in regulation of synaptic growth and function, presumably implicating a role for Futsch in mental retardation disorders.

Recently in premutation (60–200 CGG repeat bearing) male carriers of FMR1 families, a novel progressive neurodegenerative ataxia, was identified (Hagerman *et al.*, 2001). Postmortem analyses of patient tissue revealed degeneration in the cerebellum and the presence of ubiquitin-positive neuronal and glial nuclear accumulations. Jin *et al.* (2003) modeled the premutation in *Drosophila* using a human FMR1 5' UTR with premutations of 60 or 90 CGG repeat expansions upstream of the marker EGFP. Directed expression of (CGG)₆₀-EGFP and (CGG)₉₀-EGFP to the fly eye cause loss of pigmentation and progressive degeneration in an expression level and CGG repeat length-dependent manner (**Figure 13.1-2i and j; see color insert**). Thus, expression of the premutation RNA causes neurodegeneration. Expression of the CGG premutation constructs is also associated with ubiquitin- and HSP70-positive nuclear accumulations. Remarkably, upregulation of hsp70 rescues CGG-mRNA-induced neurodegeneration, whereas compromised Hsc70 activity enhances degeneration, as previously noted for polyQ protein diseases (Jin *et al.*, 2003). These data are suggestive of a common pathway between protein misfolding diseases and RNA-associated neurodegeneration.

4.3. A Model for SCA8

SCA8 is the other type II trinucleotide expansion disease for which a fly model has been generated. The CTG expansion occurs in the 3' UTR of a noncoding gene that partially overlaps with a second gene (the human homologue of *Drosophila kelch*, encoding an actin interacting protein) transcribed in the other orientation (Nemes *et al.*, 2000). Interestingly, only repeat lengths between 110 and 250 cause SCA8 symptoms, but longer (or shorter) repeats do not. One of the hypotheses concerning the mechanism of SCA8 is that this noncoding RNA may naturally act as an antisense RNA for *kelch* gene expression, with disruption of *kelch* being the cause of SCA8 pathogenesis. A second hypothesis is that CUG expanded SCA8 RNA may cause toxicity per se.

Rebay and colleagues (Mutsuddi *et al.*, 2004) used human SCA8 cDNAs (disease-length SCA8-112CTG and control SCA8-9CTG) to make a *Drosophila* model. Their detailed analysis of multiple independent lines of the constructs revealed that both the control disease gene and the pathogenic disease gene cause degenerative phenotypes (a mild external roughness and progressive internal degeneration). A loss-of-function modifier screen using the SCA8-CTG112-induced rough eye phenotype led to identification of several RNA binding proteins that modulate the phenotype, including *staufen*, *muscle-blind*, and *split ends*. Additional studies revealed that Staufen protein is recruited to the SCA8 RNA via the expanded CUG repeat sequences. It would be interesting to know whether SCA8-induced neurodegeneration is associated with other ubiquitin and stress-induced heat-shock protein positive accumulations in the fly, similar to CGG-induced neurodegeneration.

5. Modeling PD

5.1. PD

Parkinson's disease is the most common neurodegenerative movement disorder, with an incidence of 1% in the population older than 65 years of age (Lang and Lozano, 1998). The major clinical symptoms include resting tremor, rigidity, and bradykinesia (slowness in movement) due to the pro-

gressive loss of dopaminergic (DA) neurons, primarily in the substantia nigra pars compacta. Formation of Lewy bodies (LB) and Lewy neurites (LN) in the dopaminergic neurons is used for postmortem diagnosis of neuropathology in sporadic PD patients and in the rare familial forms. Although PD is mostly a sporadic disease, genetic analysis has revealed loci linked to PD or PD-like disorders, including mutations in α -synuclein, *parkin*, *ubiquitin carboxy terminal hydrolase L1* (*UCHL1*), and *DJ-1*. Mutation in α -synuclein and *UCHL1* cause autosomal dominant disease, whereas *parkin* and *DJ-1* are inherited in an autosomal recessive manner.

Impairment of mitochondrial complex I activity and subsequent oxidative stress and reduced energy level of neurons is one of the hypotheses to explain PD pathogenesis. This is supported by the findings that agents that induce oxidative damage or are complex I inhibitors, 1-methyl-4-phenyl-1, 2, 3, 6 tetrahydropyridine (MPTP), paraquat, and rotenone, lead to selective dopaminergic neuronal death (Przedborski *et al.*, 2000; Thiruchelvam *et al.*, 2000; Sherer *et al.*, 2003). Mouse and primate models using these agents show the accumulation of aggregates immunopositive for α -synuclein. In the late 1990's, two familial mutations in α -synuclein A30P and A53T were linked to dominantly inherited PD (Kruger *et al.*, 1998; Polymeropoulos *et al.*, 1997). There are several theories to explain how α -synuclein might be toxic to dopaminergic neurons. These include that abnormal polymerization of α -synuclein into filaments and the formation of LB and LN are toxic and initiate neuronal degeneration (Lee *et al.*, 2002). An alternative model is that oligomeric protofibrils of α -synuclein are the toxic entity, rather than the mature filaments (Goldberg and Lansbury, 2000). Other theories suggest that abnormal interactions of α -synuclein monomers with other proteins and subsequent inhibition of their activity may lead to neuronal dysfunction and death.

Impairment of the UPS is another major hypothesis for PD pathology. Evidence for this includes the accumulation of ubiquitinated proteins in LBs, suggesting a potential proteasomal machinery failure. Not only are LB ubiquitinated (Alves-Rodrigues *et al.*, 1998), but also it has more recently been shown that heat-shock proteins colocalize to LBs in patient tissue (Auluck *et al.*, 2002). Additional evidence came with the genetic linkage of mutations in *parkin*, encoding an E3 specific ubiquitin ligase, with a PD-like disorder (Kitada *et al.*, 1998; Leroy *et al.*, 1998). E3 ubiquitin ligases are required to transfer ubiquitin to specific target proteins for their degradation. Mutations in the *parkin* locus cause autosomal recessive juvenile onset parkinsonism (AR-JP), indicating that loss of *parkin*, and thus potentially the buildup of its target proteins, might cause disease. A rare glycosylated form of α -synuclein has been suggested to be a target of Parkin activity (Shimura *et al.*, 2001).

5.2. α -Synuclein Models for Dominant PD in the Fly

To model Parkinson disease in the fly, Feany and Bender (2000) made transgenic lines that produce normal human α -synuclein and two familial mutant forms (A30P and A53T). Expression of these transgenes was directed to the brain or DA neurons, and the overall integrity of the brain, DA neurons, LB-like accumulations, and behavioral changes were characterized. Expression of α -synuclein does not cause any obvious change in the gross morphology of even 60-day-old fly brains. However, in the same situation, there is adult onset loss of tyrosine hydroxylase (TH) immunostaining in select populations of DA neurons (TH is a DA neuronal marker). Over time, α -synuclein also forms perinuclear and neuritic filamentous inclusions remarkably similar to LBs and LNs. Flies expressing the wild-type α -synuclein, α -synuclein A30P, and α -synuclein A53T in the nervous system have reduced climbing ability, indicating that motor function may also be affected. Overexpression of α -synuclein in the eye was also reported to cause a mild retinal degeneration.

In subsequent studies, Auluck *et al.* (2002) found that directed expression of wild-type α -synuclein or mutant forms of α -synuclein (A30P and A53T) leads to selective loss of TH immunos-

taining in 50% of the dorsomedial (DM) cluster of DA neurons in flies aged to 20 days. The LB-like inclusions also immunostain for ubiquitin, as in human PD, and for the stress-induced form of Hsp70, indicating activation of a stress response (Auluck *et al.*, 2002). Takahashi *et al.* (2003) examined the phosphorylation state of α -synuclein at residue serine129 over time, to show that wild-type and pathogenic forms of α -synuclein are progressively phosphorylated in the fly similar to the situation in human PD. Phosphorylation has been shown to accelerate fibril formation of α -synuclein (Fujiwara *et al.*, 2002). Thus, phosphorylation may be important for the pathogenesis of PD, as it is for SCA1-induced polyglutamine toxicity and tau-induced neurodegeneration (see below). It will be interesting to define how mutations at Ser129 affect the pathogenicity of α -synuclein.

5.3. Parkin Models for Recessive Parkinsonism in the Fly

Two independent groups created *parkin* loss-of-function fly models by deleting the *Drosophila* homologue of the *parkin* gene (Greene *et al.*, 2003; Pesah *et al.*, 2004). In both studies, lack of activity of *parkin* in the fly did not cause loss of DA neuron integrity. However, *parkin* mutants show sensitivity to oxidative stress, and have an abnormal wing phenotype. The mutants also display impaired flight and climbing ability, leading the authors to examine the muscle morphology in detail (Greene *et al.*, 2003). They found that the flight muscles are highly degenerate and associated with swollen mitochondria, vacuoles, and an overall loss of integrity of myofibrils. These cells show features of apoptosis, including TUNEL labeling with chromatin condensation and nuclear envelope breakdown (Greene *et al.*, 2003; Pesah *et al.*, 2004). *Parkin* mutant flies also display reduced longevity and are male sterility. Although *parkin* mutant flies do not have an age-dependent DA neuron loss, these other phenotypes might still be insightful to investigate mechanisms of dysfunction upon *parkin* mutation.

5.4. Fly Modifiers of PD

The pathological similarities in protein accumulation and immunostaining with Hsp70, between polyQ and α -synuclein-mediated neurodegeneration led Auluck *et al.* (2002) to investigate whether chaperones could mitigate the DA neuronal impairment induced by α -synuclein, as chaperones do for polyQ disease. To do this, Hsp70 was coexpressed with α -synuclein, and DA neuron integrity was assessed by TH immunostaining. Indeed, directed expression of *hsp70* fully mitigates the toxicity of α -synuclein to DA neurons over time. To address the significance of chaperones in human disease, human patient tissue from PD and other synucleinopathies, including dementia with LBs (DLBs) and the LB variant of AD were examined (Auluck *et al.*, 2002). Indeed, in all situations, antibodies to the molecular chaperones Hsp70 and its cochaperone Hsp40 were found to immunolabel LBs and LNs. A role for chaperones in pathology is underscored by the finding that compromised levels of Hsp70 also accelerate α -synuclein-mediated degenerative events in flies, suggesting that a compromised stress response may contribute to human disease. Given these data in the fly, and the commonalities with human disease, chaperones may be involved in PD pathology. Indeed, recently polymorphisms in the *Hsp70-1* gene promoter that compromise activation of the gene in cell culture have been linked to PD (Wu *et al.*, 2004), and Hsp70 has been shown to regulate toxicity of α -synuclein in PD mouse models (Klucken *et al.*, 2004).

These studies were extended to a drug situation in *Drosophila* with the compound geldanamycin (GA) (Auluck and Bonini, 2002). GA is an inhibitor of Hsp90 that upregulates the activity of heat shock factor (Zou *et al.*, 1998) and mitigates polyQ aggregates in cultured mammalian

cells (Sittler *et al.*, 2001). Feeding flies GA completely suppresses degenerative events of α -synuclein *in vivo*. The protective effect of GA effect is lost if flies are deficient in heat-shock factor (hsf) activity (Auluck, 2003), indicating that GA protects DA cells through activating a stress response *in vivo*.

A microarray analysis has been done by Scherzer *et al.* (2003) on flies expressing α -synuclein, revealing genes that are up- or downregulated at early and advanced stages of toxicity. At the presymptomatic stage, expression levels of select genes are affected; these encode enzymes that regulate catecholamine and DA synthesis, lipid-binding proteins, and mitochondrial proteins involved in electron transport chain and ATP synthesis. Transcription of genes in energy metabolism is downregulated in presymptomatic stages of disease while they are upregulated in the early and advanced stages of α -synuclein pathology. Genes encoding subunits of cytochrome P450 are also upregulated at the late stages. Interestingly, there were few overlaps in gene expression changes between expression of α -synuclein and another neurodegenerative disease-associated protein tau, indicating distinct pathways lead to neuronal degeneration.

Parkin-associated endothelin-like receptor (Pael-R) is a G protein-coupled transmembrane polypeptide (Imai *et al.*, 2001) that interacts with Parkin in yeast two-hybrid assays. Parkin ubiquitinates Pael-R, which prevents its accumulation and subsequent cell death induced by endoplasmic reticulum stress in cell culture. Thus, Pael-R is a potential parkin substrate that might contribute to DA neuronal degeneration in AR-JP. In flies, directed expression of Pael-R impairs DA neuron integrity, which is enhanced by reduction of Parkin levels and suppressed by upregulation of Parkin or expression of human Parkin (Yang *et al.*, 2003). The role of Pael-R in human PD is still under investigation. Parkin is also found to be present in LBs in PD and DLB (Shimura *et al.*, 2001; Schlossmacher *et al.*, 2002), suggesting that α -synuclein might be one of Parkin substrates. Indeed, upregulation of *parkin* suppresses α -synuclein-induced loss of DA neuron integrity *in vivo* in the fly (Yang *et al.*, 2003). Additionally, Haywood and Staveley (2004) found that Parkin also suppresses other putative phenotypes of α -synuclein, including retinal degeneration and loss of climbing ability. Therefore, studying PD phenotypes in the fly has revealed functional interactions between different genes involved in parkinsonism, and pioneered new avenues toward treatment.

5.5. Modeling PD in *C. elegans*

Both α -synuclein and drug-induced models of PD have been generated in the nematode. To make an α -synuclein model in the nematode, Lasko *et al.* (2003) generated transgenic lines that express normal and mutant forms of α -synuclein pan-neuronally, as well as in DA neurons and motor neurons. Significant reduction of DA neuron cell bodies and dendritic processes, visualized by expression of GFP, were seen upon expression pan-neuronally or in DA neurons, but not with motor neuron-specific expression. Unlike fly α -synuclein PD models, the neuronal defects in the nematode were not progressive over time. Functional studies show that α -synuclein expressed in a pan-neural pattern or in motor neurons compromises head-thrashing movement.

Nass and colleagues (Nass *et al.*, 2002) used treatment with 6-hydroxydopamine (6-OHDA) to create a neurotoxin-induced model of parkinsonism. Exposure of larvae to 6-OHDA causes morphological changes and complete loss of marker GFP expression in many of the cell bodies of DA neurons, but with no effect on serotonergic, cholinergic, and chemosensory neurons. This toxicity is mitigated by coexposure of animals to inhibitors of the dopamine transporter, which is required for uptake of 6-OHDA. Ultrastructural analysis revealed that in affected cells, the majority of dendritic endings are shrunken and associated with vacuoles. Despite the fact that the morphology of the dying

cells resembles programmed cell death, animals lacking *ced-3* or *ced-4* gene activities, which are essential for programmed cell death, show similar sensitivity to 6-OHDA.

6. Modeling Alzheimer's and Related Diseases

6.1. Alzheimer's and Related Diseases

Alzheimer's disease is the most common neurodegenerative disease in the elderly, associated with impairment in cognition and progressive loss of memory (Price *et al.*, 1998). Familial forms of AD have been found, with mutations in the genes that encode amyloid precursor protein (APP) and Presenilin 1 and 2 (PS1 and PS2). Alzheimer's disease selectively damages brain regions and neuronal circuits critical for cognition and memory in the neocortex, hippocampus, amygdala, and cholinergic neurons. One of the pathological features of AD is that many affected neurons have extracellular filamentous proteinaceous aggregates called senile plaques. These contain A β peptides (A β 40 and mainly A β 42) generated from the proteolytic processing of APP via γ -secretase complex, of which PS1 and PS2 are subunits, and β -secretase/BACE (Hardy and Selkoe, 2002; Driscoll and Gerstbrein, 2003; Wilson *et al.*, 2003). Alzheimer's disease is also associated with neurofibrillary tangles (NFT) in cell bodies, dendrites, and axons that consist of hyperphosphorylated tau.

In addition to hyperphosphorylated tau being the major component of neurofibrillary tangles in AD and other tauopathies such as progressive supranuclear palsy, corticobasal degeneration, Pick's disease, and argyrophilic grain disease, several mutations in the tau gene have been linked to frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (Lee *et al.*, 2001). Tau is expressed in the nervous system, and plays a role in assembly and stabilization of microtubules. Human brain has six tau isoforms: three with three repeats of the microtubule binding domain, and three with four repeats of the microtubule binding domain. Mutations are concentrated around the microtubule binding repeats. Analysis of the missense, deletion mutations, silent mutations in the coding region, or the mutations in the intronic region of the *tau* gene indicates that several of the mutations change the ratio of four repeat to three repeat tau isoforms. *Drosophila* has one human tau homologue that expresses highly in the brain and eye (Heidary and Fortini, 2001).

6.2. Modeling AD with A β and APP in *Drosophila*

Flies have some components of γ -secretase activity including presenilin (Psn) and nicastrin, but the fly APP lacks the A β domain and the fly has no BACE activity (Kopan and Goate, 2002; Bonini and Fortini, 2003; Driscoll and Gerstbrein, 2003). Overexpression of Psn and human familial forms associated with AD cause programmed cell death in flies, with the mutant forms having reduced toxicity compared to the normal protein (Ye and Fortini, 1999).

To gain insight into the physiological function of wild-type human APP (*hAPP*), pathogenic APP mutations, and the *Drosophila* homologue of APP (*APPL*, for *APP-like*), Fossgreen *et al.* (1998) expressed these genes in insect cell culture and in various fly tissues to characterize the phenotypes associated with overexpression. The only noticeable phenotype was a blistered-wing when *hAPP*, AD-associated mutation-*hAPP*-Swedish and *dAPPL* were expressed in the developing wing tissue, suggesting a shared function of APP family members in cell-cell adhesion. In a subsequent study (Yagi *et al.*, 2000), directed expression of the *hAPP* gene lead to localization of *hAPP* at synaptic terminals in the nervous system, similar to endogenous *dAPPL* localization. Interestingly, *dAPPL* has been found in ThioflavinS positive, a dye specific for amyloid structure, protein aggregates formed

in the fly mutant *blue cheese*, which causes progressive neurodegeneration and a shortened life span (Finley *et al.*, 2003).

To test the pathogenicity of the peptides A β 42 and A β 40, Iijima *et al.* (2004) directed the expression of human A β 40 and A β 42 to the nervous system. Both A β 42 and A β 40 cause a progressive loss of learning ability. However, only A β 42 but not A β 40 reduces the climbing ability and life span of the flies, and induces degeneration in Kenyon cells in the brain. Directed expression of A β 42 also forms diffuse accumulations reminiscent of amyloid plaques, which stain with ThioflavinS. Ultrastructural analysis of degenerating Kenyon cells showed features of necrotic cell death with swollen mitochondria.

As noted above, the *Drosophila* homologue of hAPP, *dAPPL* lacks the A β region from which A β 40/42 are derived, and *Drosophila* also lacks the enzyme BACE (β -secretase) which is required along with gamma-secretase for proteolytic processing of hAPP to generate A β . Therefore, to create an APP fly model, Greeve *et al.* (2004) made transgenics that express both hAPP and human BACE. In this situation, directed expression of hAPP and human BACE in the eye leads to production of A β 40/42 fragments, accompanied by progressive degeneration of retinal structure. Moreover, large globular protein deposits positively stained by thioflavinS or β -amyloid-specific antibody indicates that amyloid-like proteinaceous "senile plaques" also form in the fly, although whether these are a consequence of or proceed degeneration is not clear. Overexpression of Psn to enhance γ -secretase activity enhances the neurodegeneration. Ubiquitous expression of hAPP, hBACE, and *Drosophila* Psn also causes an ectopic wing vein phenotype that is suppressed by loss-of-function mutations in presenilin activity. Moreover, BACE inhibitors mitigate the phenotype, indicating that processing of hAPP is required for neurodegeneration.

To investigate whether APP family members function in vesicle trafficking and the role of this process to Alzheimer is disease pathology, Gunawardena and Goldstein (2001) directed the expression of hAPP, two human familial mutant forms (*hAPP-LONDON* and *hAPP-Swedish*) and the *Drosophila dAPPL* to the larval neurons and analyzed the extent of organelle accumulation in axons. They found that overexpression of hAPP and *dAPPL* cause organelle jams that are strikingly similar to those induced upon loss of function of motor proteins like kinesin. This phenotype is further enhanced by 50% reduction of kinesin subunits involved in anterograde transport to the nerve terminal or suppressed by reduction in dynein subunits required for the retrograde transport to the nerve body. Interestingly, the authors also reported TUNEL-positive programmed cell death of the larval neurons upon overexpression of APP family members, which required both the C-terminal kinesin interaction domain and the A β region. Thus, *Drosophila* APP induces axonal blockages, but not TUNEL labeling of neurons.

6.3. Tau Models in *Drosophila*

Expression of tau in flies causes problems with axonal transport, axonal pathways, and degeneration (Torroja *et al.*, 1999; Wittmann *et al.*, 2001; Jackson *et al.*, 2002; Kraemer *et al.*, 2003; Nishimura *et al.*, 2004). Expression of both normal and human disease-associated mutant forms of *tau* in the nervous system of the fly decreases life span, with the mutant form having more profound effects. Aged flies exhibit neurodegeneration in the cortex that is often associated with vacuolization. This degeneration is progressive, and again is more severe for pathogenic tau. Expression selectively directed to cholinergic neurons (the cell type predominantly affected in human disease) also causes vacuolization and loss of cholinergic neurons. Directed expression in the eye gives a severe rough eye phenotype, indicating that toxicity of tau is relatively broad (Figure 13.1-2g; see color insert). Phosphorylation of tau and accumulation of tau in select areas of degenerating neurons are seen, but

EM analysis of degenerating neurons does not show any obvious filamentous aggregates, indicating that neurodegeneration may not require formation of “tangle-like” accumulations in the fly.

6.4. Modifiers of AD and Tauopathies in the Fly

Given that hyperphosphorylated tau is the major component of neurofibrillary tangles, several researchers have looked at the influences of kinases on tau pathology. Serine–threonine kinase, GSK-3 β , is one of the candidate kinases that might be involved in phosphorylation of tau in human brain in AD or other tauopathies (Sperber *et al.*, 1995; Mandelkow, 1999; Lee *et al.*, 2001). Jackson *et al.* (2002) tested the *Drosophila* homologue of human GSK-3 β , *shaggy/zeste white-3*, also a component of wingless signaling pathway, for interactions with tau. Interestingly, they found loss of function of *shaggy* suppresses the tau-induced rough eye phenotype, whereas overexpression of *shaggy* both enhances tau toxicity and leads to the formation of neurofibrillary tangle-like accumulations. Jackson *et al.* (2002) also found that directed expression of antiapoptotic genes, including DIAP1, DIAP2, and p35, have a mild suppression effect, indicating that the cells may be dying in part through apoptosis (**Figure 13.1-2g and h; see color insert**). DIAP1 was also identified in a genetic screen for modifiers of tau (Shulman and Feany, 2003).

In an overexpression screen for *in vivo* modifiers of the tau-mediated rough eye phenotype, kinases and phosphatases were found as the major group of modifier genes (Shulman and Feany, 2003). In addition to these, and genes involved in apoptosis, components of the cytoskeleton, and cation transporters were also found. Among the modifiers, two of them were quite interesting, which may link two other human neurodegenerative diseases to pathology. Overexpression of *dfmr/dfx1* and the *Drosophila* homologue of the SCA2 gene apparently enhance tau eye degeneration. In addition, modifiers of SCA1 toxicity fail to show an interaction with tau. This suggests that modifiers of polyQ toxicity and those of tau are distinct.

To further address the functional role of phosphorylation in tau toxicity, Nishimura *et al.* (2004) investigated in detail the function of *Drosophila* *PAR-1* gene, which was also independently identified by Shulman and Feany (2003) as an enhancer of tau toxicity. Directed expression of *Par-1* in the eye causes a dose-dependent rough eye phenotype and loss of photoreceptor neurons. Interestingly, PAR-1-induced eye degeneration is suppressed by a deletion line that uncovers *Drosophila* *tau*, whereas coexpression of *PAR-1* and human *tau* synergistically enhance degeneration. Using several phosphorylation-specific antibodies, tau appears to be phosphorylated at conserved S262/S356 sites by PAR-1. To test the significance of this phosphorylation, these sites were mutated to alanines (A) to prevent phosphorylation and then the toxicity of the protein was analyzed *in vivo*. Directed expression of *tau-S262A/S356A* has strikingly reduced toxicity, indicating that tau phosphorylation is critical to the toxicity of the protein *in vivo* (Nishimura *et al.*, 2004).

6.5. Modeling Alzheimer's and Related Diseases in C. elegans

The first invertebrate model of human neurodegenerative disease in the nematode was made by directing expression of human A β 42 to muscle (Link, 1995). Immunohistochemical analysis of these animals revealed intracellular deposits that stain for amyloid-specific antibodies. These deposits are also thioflavinS positive, and coexpression of transthyretin, which inhibits amyloid deposits, leads to significant reduction in the number of dye-positive accumulations. Link and colleagues also examined the temporal relationship between A β 42 expression and oxidative stress (Drake *et al.*, 2003). Animals expressing human A β 42 in a temperature-inducible manner show a progressive increase in oxidative stress as measured by protein carbonylation. Temperature-inducible A β 42 expressing nema-

todes do not have amyloid deposits, indicating that changes in oxidative status precede amyloid formation.

Kraemer *et al.* (2003) generated a *C. elegans* model for tauopathies using wild-type and human disease-associated mutant forms of tauP301L and tauV337M. Expression of all forms reduced life span and caused progressive uncoordinated movement, although the pathogenic mutant forms were more severe than the wild type. Here, axonal degeneration of neurons was also associated with vacuoles, membranous infoldings, and tau-positive accumulations detected by EM analysis. Phosphorylation of tau also occurred *in vivo*, but did not correlate with toxicity.

6.6. *C. elegans* Modifiers of AD Phenotypes

To find modifiers of A β toxicity, Fonte *et al.* (2002) took a biochemical approach. They found six proteins that specifically coimmunoprecipitated with antibodies to A β 42 but not with a control GFP antibody. Mass spectrometric analysis of these proteins revealed two Hsp70 family members, three small heat-shock proteins, and a tetratricopeptide repeat containing protein—remarkable for their previous association with polyQ toxicity in *Drosophila*. RNAi inhibition of the tetratricopeptide repeat containing gene-suppressed A β 42 toxicity, presumably by upregulating heat-shock proteins. A larger scale gene expression analysis has been done to define expression changes associated with A β 42 toxicity in the nematode (Link *et al.*, 2003). Whereas 67 genes were upregulated, about 240 were downregulated. The link to human disease was made by examining whether similar gene expression changes of select genes also occurred in human AD tissue. Interestingly, the transcript levels of α B-crystallin and tumor necrosis factor-induced protein 1 are induced in both the nematode model and AD patient brain, underscoring the value of invertebrate models to reveal new insight into human disease.

7. Future Directions

Taken together, these studies provide a compelling argument that *Drosophila* and *C. elegans* models for human neurodegenerative diseases can provide new insight into mechanisms of degeneration and open new avenues toward cures. The range of diseases studied has accelerated since the first polyQ model in 1998, such that now there exist models for all the major human neurodegenerative diseases. A similar approach can equally well be applied to other human diseases not neurodegenerative in nature. These studies incorporate genetics, genomics, as well as pharmaceutical approaches, to provide proof of concept and the foundation for similar approaches in more complex and expensive systems. The pace of this research is not likely to slacken. Moreover, in addition to fly and nematode models, other organisms have insight to provide. For example, yeast is proving a powerful model system for uncovering complementary aspects of the biology of human neurodegenerative diseases. In an elegant study by Outeiro and Lindquist (2003), they revealed new insight and modulatory genes of relevance to α -synuclein phenotypes and toxicity. Yeast has also been used to define polyQ modifiers, the majority of which belong to the stress response, ubiquitin-dependent proteolytic pathways and protein folding pathways (Willingham *et al.*, 2003), underscoring the critical importance of these pathways, highlighted above. Moreover, by now, the potential contribution of chaperone depletion to disease and/or the ability of upregulation of chaperones to mitigate neurodegeneration as first shown *in vivo* in the fly has been seen for a number of polyQ diseases, α -synuclein-induced toxicity, and even amyotrophic lateral sclerosis (Cummings *et al.*, 2001; Adachi *et al.*, 2003; Hay *et al.*, 2004; Kieran *et al.*, 2004). Given the task at hand—to open new avenues to the understanding and potential treatment of human neurodegenerative diseases—each organism will have a contribution to make.

Ultimately, it is the synergy of these systems to provide insight into the gold standard—human disease—that will open new pathways toward potential cures.

8. Abbreviations

A β	Amyloid β peptide
AD	Alzheimer's disease
APP	Amyloid precursor protein
AR-JP	Autosomal recessive juvenile onset parkinsonism
CBP	CREB-binding protein
DA	Dopamine, dopaminergic
DLB	Dementia with LBs
DM	Dorsomedial
dMLF	Myeloid leukemia factor 1
DRPLA	Dentatorubral-pallidoluysian atrophy
FTDP	Frontotemporal dementia with parkinsonism
FTDP-17	Frontotemporal dementia and parkinsonism linked to chromosome 17
GA	Geldanamycin
GST	Glutathione-S transferase
hAR-Q52	Pathogenic form of the human androgen receptor
HD	Huntington's disease
HDAC	Histone deacetylase
hsf	Heat-shock transcription factor
hsp40	Heat-shock protein 40
hsp70	Heat-shock protein 70
LB	Lewy body
LN	Lewy neurite
MPTP	1-Methyl-4-phenyl-1, 2, 3, 6 tetrahydropyridine
mTOR	Mammalian target of rapamycin
NFT	Neurofibrillary tangles
6-OHDA	6-Hydroxydopamine
Pael-R	Parkin-associated endothelin like receptor
P/CAF	p300/CBP-associated factor
PD	Parkinson's disease
PQE-1	PolyQ enhancer protein
PS1	Presenilin 1
PS2	Presenilin 2
Psn	Presenilin
SCA	Spinocerebellar ataxia
SBMA	Spinobulbar muscular atrophy
MJD/SCA3	Machado Joseph disease/spinocerebellar ataxia type 3
SCA3tr-Q27	C-terminal portion of human SCA3 with a normal length polyglutamine repeat of Q27
SCA3tr-Q78	C-terminal portion of human SCA3 with a disease-associated expanded polyglutamine repeat of Q78
SCA1-Q30	SCA1 protein with a control length polyQ repeat
TH	Tyrosine hydroxylase

UPS ubiquitin proteasomal system
Htt^{exon1} Huntingtin exon1 protein

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References

- Adachi, H., Katsuno, M., Minamiyama, M., Sang, C., Pagoulatos, G., Angelidis, C., Kusakabe, M., Yoshiki, A., Kobayashi, Y., Doyu, M., and Sobue, G. (2003). Heat shock protein 70 chaperone overexpression ameliorates phenotypes of the spinal and bulbar muscular atrophy transgenic mouse model by reducing nuclear-localized mutant androgen receptor protein. *J. Neurosci.* 23:2203–2211.
- Alves-Rodrigues, A., Gregori, L., and Figueiredo-Pereira, M.E. (1998). Ubiquitin, cellular inclusions and their role in neurodegeneration. *Trends Neurosci.* 21:516–520.
- Auluck, P.K. (2003). Chaperone prevention of Parkinson's disease in *Drosophila*. Dissertation Thesis, University of Pennsylvania, Philadelphia.
- Auluck, P.K., and Bonini, N.M. (2002). Pharmacological prevention of Parkinson disease in *Drosophila*. *Nat. Med.* 8:1185–1186.
- Auluck, P.K., Chan, H.Y., Trojanowski, J.Q., Lee, V.M., and Bonini, N.M. (2002). Chaperone suppression of alpha-synuclein toxicity in a *Drosophila* model for Parkinson's disease. *Science* 295:865–868.
- Bargmann, C.I. (1998). Neurobiology of the *Caenorhabditis elegans* genome. *Science* 282:2028–2033.
- Bonini, N.M., and Fortini, M.E. (2003). Human neurodegenerative disease modeling using *Drosophila*. *Annu. Rev. Neurosci.* 26:627–656.
- Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118:401–415.
- Bukau, B., and Horwich, A.L. (1998). The Hsp70 and Hsp60 chaperone machines. *Cell* 92:351–366.
- Burright, E.N., Clark, H.B., Servadio, A., Matilla, T., Feddersen, R.M., Yunis, W.S., Duvick, L.A., Zoghbi, H.Y., and Orr, H.T. (1995). SCA1 transgenic mice: a model for neurodegeneration caused by an expanded CAG trinucleotide repeat. *Cell* 82:937–948.
- Chai, Y., Koppenhafer, S.L., Shoesmith, S.J., Perez, M.K., and Paulson, H.L. (1999). Evidence for proteasome involvement in polyglutamine disease: localization to nuclear inclusions in SCA3/MJD and suppression of polyglutamine aggregation in vitro. *Hum. Mol. Genet.* 8:673–682.
- Chan, H.Y., Warrick, J.M., Gray-Board, G.L., Paulson, H.L., and Bonini, N.M. (2000). Mechanisms of chaperone suppression of polyglutamine disease: selectivity, synergy and modulation of protein solubility in *Drosophila*. *Hum. Mol. Genet.* 9:2811–2820.
- Chan, H.Y., Warrick, J.M., Andriola, I., Merry, D., and Bonini, N.M. (2002). Genetic modulation of polyglutamine toxicity by protein conjugation pathways in *Drosophila*. *Hum. Mol. Genet.* 11:2895–2904.
- Chen, H.K., Fernandez-Funez, P., Acevedo, S.F., Lam, Y.C., Kaytor, M.D., Fernandez, M.H., Aitken, A., Skoulakis, E.M., Orr, H.T., Botas, J., and Zoghbi, H.Y. (2003). Interaction of Akt-phosphorylated ataxin-1 with 14-3-3 mediates neurodegeneration in spinocerebellar ataxia type 1. *Cell* 113:457–468.
- Cummings, C.J., and Zoghbi, H.Y. (2000). Fourteen and counting: unraveling trinucleotide repeat diseases. *Hum. Mol. Genet.* 9:909–916.
- Cummings, C.J., Mancini, M.A., Antalffy, B., DeFranco, D.B., Orr, H.T., and Zoghbi, H.Y. (1998). Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1. *Nat. Genet.* 19:148–154.
- Cummings, C.J., Sun, Y., Opal, P., Antalffy, B., Mestrlil, R., Orr, H.T., Dillmann, W.H., and Zoghbi, H.Y. (2001). Overexpression of inducible HSP70 chaperone suppresses neuropathology and improves motor function in SCA1 mice. *Hum. Mol. Genet.* 10:1511–1518.

- Dockendorff, T.C., Su, H.S., McBride, S.M., Yang, Z., Choi, C.H., Siwicki, K.K., Sehgal, A., and Jongens, T.A. (2002). *Drosophila* lacking *dfmrl* activity show defects in circadian output and fail to maintain courtship interest. *Neuron* 34:973–984.
- Drake, J., Link, C.D., and Butterfield, D.A. (2003). Oxidative stress precedes fibrillar deposition of Alzheimer's disease amyloid beta-peptide (1–42) in a transgenic *Caenorhabditis elegans* model. *Neurobiol. Aging* 24:415–420.
- Driscoll, M., and Gerstbrein, B. (2003). Dying for a cause: invertebrate genetics takes on human neurodegeneration. *Nat. Rev. Genet.* 4:181–194.
- Emamian, E.S., Kaytor, M.D., Duvick, L.A., Zu, T., Tousey, S.K., Zoghbi, H.Y., Clark, H.B., and Orr, H.T. (2003). Serine 776 of ataxin-1 is critical for polyglutamine-induced disease in SCA1 transgenic mice. *Neuron* 38:375–387.
- Faber, P.W., Alter, J.R., MacDonald, M.E., and Hart, A.C. (1999). Polyglutamine-mediated dysfunction and apoptotic death of a *Caenorhabditis elegans* sensory neuron. *Proc. Natl. Acad. Sci. USA* 96:179–184.
- Faber, P.W., Voisine, C., King, D.C., Bates, E.A., and Hart, A.C. (2002). Glutamine/proline-rich PQE-1 proteins protect *Caenorhabditis elegans* neurons from huntingtin polyglutamine neurotoxicity. *Proc. Natl. Acad. Sci. USA* 99:17131–17136.
- Feany, M.B., and Bender, W.W. (2000). A *Drosophila* model of Parkinson's disease. *Nature* 404:394–398.
- Fernandez-Funez, P., Nino-Rosales, M.L., de Gouyon, B., She, W.C., Luchak, J.M., Martinez, P., Turiegano, E., Benito, J., Capovilla, M., Skinner, P.J., McCall, A., Canal, I., Orr, H.T., Zoghbi, H.Y., and Botas, J. (2000). Identification of genes that modify ataxin-1-induced neurodegeneration. *Nature* 408:101–106.
- Finley, K.D., Edeen, P.T., Cumming, R.C., Mardahl-Dumesnil, M.D., Taylor, B.J., Rodriguez, M.H., Hwang, C.E., Benedetti, M., and McKeown, M. (2003). Blue cheese mutations define a novel, conserved gene involved in progressive neural degeneration. *J. Neurosci.* 23:1254–1264.
- Fonte, V., Kapulkin, V., Taft, A., Fluet, A., Friedman, D., and Link, C.D. (2002). Interaction of intracellular beta amyloid peptide with chaperone proteins. *Proc. Natl. Acad. Sci. USA* 99:9439–9444.
- Fortini, M.E., and Bonini, N.M. (2000). Modeling human neurodegenerative diseases in *Drosophila*: on a wing and a prayer. *Trends Genet.* 16:161–167.
- Fossgreen, A., Bruckner, B., Czech, C., Masters, C.L., Beyreuther, K., and Paro, R. (1998). Transgenic *Drosophila* expressing human amyloid precursor protein show gamma-secretase activity and a blistered-wing phenotype. *Proc. Natl. Acad. Sci. USA* 95:13703–13708.
- Fujiwara, H., Hasegawa, M., Dohmae, N., Kawashima, A., Masliah, E., Goldberg, M.S., Shen, J., Takio, K., and Iwatsubo, T. (2002). alpha-Synuclein is phosphorylated in synucleinopathy lesions. *Nat. Cell Biol.* 4:160–164.
- Gao, F.B. (2002). Understanding fragile X syndrome: insights from retarded flies. *Neuron* 34:859–862.
- Goldberg, M.S., and Lansbury, P.T., Jr. (2000). Is there a cause-and-effect relationship between alpha-synuclein fibrillogenesis and Parkinson's disease? *Nat. Cell Biol.* 2:E115–E119.
- Grams, R., and Korge, G. (1998). The *mub* gene encodes a protein containing three KH domains and is expressed in the mushroom bodies of *Drosophila melanogaster*. *Gene* 215:191–201.
- Green, H. (1993). Human genetic diseases due to codon reiteration: relationship to an evolutionary mechanism. *Cell* 74:955–956.
- Greene, J.C., Whitworth, A.J., Kuo, I., Andrews, L.A., Feany, M.B., and Pallanck, L.J. (2003). Mitochondrial pathology and apoptotic muscle degeneration in *Drosophila* parkin mutants. *Proc. Natl. Acad. Sci. USA* 100:4078–4083.
- Greeve, I., Kretschmar, D., Tschape, J.A., Beyn, A., Brellinger, C., Schweizer, M., Nitsch, R.M., and Reifegerste, R. (2004). Age-dependent neurodegeneration and Alzheimer-amyloid plaque formation in transgenic *Drosophila*. *J. Neurosci.* 24:3899–3906.
- Gunawardena, S., and Goldstein, L.S. (2001). Disruption of axonal transport and neuronal viability by amyloid precursor protein mutations in *Drosophila*. *Neuron* 32:389–401.
- Gunawardena, S., Her, L.S., Bruschi, R.G., Laymon, R.A., Niesman, I.R., Gordesky-Gold, B., Sintasath, L., Bonini, N.M., and Goldstein, L.S. (2003). Disruption of axonal transport by loss of huntingtin or expression of pathogenic polyQ proteins in *Drosophila*. *Neuron* 40:25–40.
- Hagerman, R.J., Leehey, M., Heinrichs, W., Tassone, F., Wilson, R., Hills, J., Grigsby, J., Gage, B., and Hagerman, P.J. (2001). Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. *Neurology* 57:127–130.
- Hardy, J., and Selkoe, D.J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297:353–356.
- Hay, D.G., Sathasivam, K., Tobaben, S., Stahl, B., Marber, M., Mestrl, R., Mahal, A., Smith, D.L., Woodman, B., and Bates, G.P. (2004). Progressive decrease in chaperone protein levels in a mouse model of Huntington's disease and induction of stress proteins as a therapeutic approach. *Hum. Mol. Genet.* 13:1389–1405.

- Haywood, A.F., and Staveley, B.E. (2004). parkin counteracts symptoms in a *Drosophila* model of Parkinson's disease. *BMC Neurosci.* 5:14.
- Heidary, G., and Fortini, M.E. (2001). Identification and characterization of the *Drosophila* tau homolog. *Mech. Dev.* 108:171–178.
- Higashiyama, H., Hirose, F., Yamaguchi, M., Inoue, Y.H., Fujikake, N., Matsukage, A., and Kakizuka, A. (2002). Identification of ter94, *Drosophila* VCP, as a modulator of polyglutamine-induced neurodegeneration. *Cell Death Differ.* 9:264–273.
- Hsu, A.L., Murphy, C.T., and Kenyon, C. (2003). Regulation of aging and age-related disease by DAF-16 and heat-shock factor. *Science* 300:1142–1145.
- Iijima, K., Liu, H.P., Chiang, A.S., Hearn, S.A., Konsolaki, M., and Zhong, Y. (2004). Dissecting the pathological effects of human Abeta40 and Abeta42 in *Drosophila*: a potential model for Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* 101:6623–6628.
- Ikeda, H., Yamaguchi, M., Sugai, S., Aze, Y., Narumiya, S., and Kakizuka, A. (1996). Expanded polyglutamine in the Machado-Joseph disease protein induces cell death in vitro and in vivo. *Nat. Genet.* 13:196–202.
- Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y., and Takahashi, R. (2001). An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell* 105:891–902.
- Jackson, G.R., Salecker, I., Dong, X., Yao, X., Arnheim, N., Faber, P.W., MacDonald, M.E., and Zipursky, S.L. (1998). Polyglutamine-expanded human huntingtin transgenes induce degeneration of *Drosophila* photoreceptor neurons. *Neuron* 21:633–642.
- Jackson, G.R., Wiedau-Pazos, M., Sang, T.K., Wagle, N., Brown, C.A., Massachi, S., and Geschwind, D.H. (2002). Human wild-type tau interacts with wingless pathway components and produces neurofibrillary pathology in *Drosophila*. *Neuron* 34:509–519.
- Jana, N.R., and Nukina, N. (2003). Recent advances in understanding the pathogenesis of polyglutamine diseases: involvement of molecular chaperones and ubiquitin-proteasome pathway. *J. Chem. Neuroanat.* 26:95–101.
- Jin, P., Zarnescu, D.C., Zhang, F., Pearson, C.E., Lucchesi, J.C., Moses, K., and Warren, S.T. (2003). RNA-mediated neurodegeneration caused by the fragile X premutation rCGG repeats in *Drosophila*. *Neuron* 39:739–747.
- Katsuno, M., Adachi, H., Kume, A., Li, M., Nakagomi, Y., Niwa, H., Sang, C., Kobayashi, Y., Doyu, M., and Sobue, G. (2002). Testosterone reduction prevents phenotypic expression in a transgenic mouse model of spinal and bulbar muscular atrophy. *Neuron* 35:843–854.
- Kazantsev, A., Walker, H.A., Slepko, N., Bear, J.E., Preisinger, E., Steffan, J.S., Zhu, Y.Z., Gertler, F.B., Housman, D.E., Marsh, J.L., and Thompson, L.M. (2002). A bivalent Huntingtin binding peptide suppresses polyglutamine aggregation and pathogenesis in *Drosophila*. *Nat. Genet.* 30:367–376.
- Kazemi-Esfarjani, P., and Benzer, S. (2000). Genetic suppression of polyglutamine toxicity in *Drosophila*. *Science* 287:1837–1840.
- Kazemi-Esfarjani, P., and Benzer, S. (2002). Suppression of polyglutamine toxicity by a *Drosophila* homolog of myeloid leukemia factor 1. *Hum. Mol. Genet.* 11:2657–2672.
- Kieran, D., Kalmar, B., Dick, J.R., Riddoch-Contreras, J., Burnstock, G., and Greensmith, L. (2004). Treatment with arimocloamol, a coinducer of heat shock proteins, delays disease progression in ALS mice. *Nat. Med.* 10:402–405.
- Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 392:605–608.
- Klucken, J., Shin, Y., Masliah, E., Hyman, B.T., and McLean, P.J. (2004). Hsp70 reduces alpha-synuclein aggregation and toxicity. *J. Biol. Chem.* 279:25497–25502.
- Kopan, R., and Goate, A. (2002). Aph-2/Nicastrin: an essential component of gamma-secretase and regulator of Notch signaling and Presenilin localization. *Neuron* 33:321–324.
- Kraemer, B.C., Zhang, B., Leverenz, J.B., Thomas, J.H., Trojanowski, J.Q., and Schellenberg, G.D. (2003). Neurodegeneration and defective neurotransmission in a *Caenorhabditis elegans* model of tauopathy. *Proc. Natl. Acad. Sci. USA* 100:9980–9985.
- Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J.T., Schols, L., and Riess, O. (1998). Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nat. Genet.* 18:106–108.
- Lakso, M., Vartiainen, S., Moilanen, A.M., Sirvio, J., Thomas, J.H., Nass, R., Blakely, R.D., and Wong, G. (2003). Dopaminergic neuronal loss and motor deficits in *Caenorhabditis elegans* overexpressing human alpha-synuclein. *J. Neurochem.* 86:165–172.
- Lang, A.E., and Lozano, A.M. (1998). Parkinson's disease. First of two parts. *N. Engl. J. Med.* 339:1044–1053.

- La Spada, A.R., Wilson, E.M., Lubahn, D.B., Harding, A.E., and Fischbeck, K.H. (1991). Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature* 352:77–79.
- Lee, M.K., Stirling, W., Xu, Y., Xu, X., Qui, D., Mandir, A.S., Dawson, T.M., Copeland, N.G., Jenkins, N.A., and Price, D.L. (2002). Human alpha-synuclein-harboring familial Parkinson's disease-linked Ala-53 → Thr mutation causes neurodegenerative disease with alpha-synuclein aggregation in transgenic mice. *Proc. Natl. Acad. Sci. USA* 99: 8968–8973.
- Lee, V.M., Goedert, M., and Trojanowski, J.Q. (2001). Neurodegenerative tauopathies. *Annu. Rev. Neurosci.* 24: 1121–1159.
- Lee, W.C., Yoshihara, M., and Littleton, J.T. (2004). Cytoplasmic aggregates trap polyglutamine-containing proteins and block axonal transport in a *Drosophila* model of Huntington's disease. *Proc. Natl. Acad. Sci. USA* 101:3224–3229.
- Leroy, E., Boyer, R., Auburger, G., Leube, B., Ulm, G., Mezey, E., Harta, G., Brownstein, M.J., Jonnalagada, S., Chernova, T., Dehejia, A., Lavedan, C., Gasser, T., Steinbach, P.J., Wilkinson, K.D., and Polymeropoulos, M.H. (1998). The ubiquitin pathway in Parkinson's disease. *Nature* 395:451–452.
- Link, C.D. (1995). Expression of human beta-amyloid peptide in transgenic *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* 92:9368–9372.
- Link, C.D., Taft, A., Kapulkin, V., Duke, K., Kim, S., Fei, Q., Wood, D.E., and Sahagan, B.G. (2003). Gene expression analysis in a transgenic *Caenorhabditis elegans* Alzheimer's disease model. *Neurobiol. Aging* 24:397–413.
- Mandelkow, E. (1999). Alzheimer's disease: the tangled tale of tau. *Nature* 402:588–589.
- Mangiarini, L., Sathasivam, K., Seller, M., Cozens, B., Harper, A., Hetherington, C., Lawton, M., Trotter, Y., Leach, H., Davies, S.W., and Bates, G.P. (1996). Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. *Cell* 87:493–506.
- Marsh, J.L., Walker, H., Theisen, H., Zhu, Y.Z., Fielder, T., Purcell, J., and Thompson, L.M. (2000). Expanded polyglutamine peptides alone are intrinsically cytotoxic and cause neurodegeneration in *Drosophila*. *Hum. Mol. Genet.* 9:13–25.
- Mathew, A., and Morimoto, R.I. (1998). Role of the heat-shock response in the life and death of proteins. *Ann. N. Y. Acad. Sci.* 851:99–111.
- Morales, J., Hiesinger, P.R., Schroeder, A.J., Kume, K., Verstreken, P., Jackson, F.R., Nelson, D.L., and Hassan, B.A. (2002). *Drosophila* fragile X protein, DFXR, regulates neuronal morphology and function in the brain. *Neuron* 34:961–972.
- Morley, J.F., Brignull, H.R., Weyers, J.J., and Morimoto, R.I. (2002). The threshold for polyglutamine-expansion protein aggregation and cellular toxicity is dynamic and influenced by aging in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* 99:10417–10422.
- Mutsaers, M., Marshall, C.M., Benzow, K.A., Koob, M.D., and Rebay, I. (2004). The spinocerebellar ataxia 8 noncoding RNA causes neurodegeneration and associates with stauin in *Drosophila*. *Curr. Biol.* 14:302–308.
- Nass, R., Hall, D.H., Miller, D.M., 3rd, and Blakely, R.D. (2002). Neurotoxin-induced degeneration of dopamine neurons in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* 99:3264–3269.
- Nemes, J.P., Benzow, K.A., Moseley, M.L., Ranum, L.P., and Koob, M.D. (2000). The SCA8 transcript is an antisense RNA to a brain-specific transcript encoding a novel actin-binding protein (KLHL1). *Hum. Mol. Genet.* 9:1543–1551.
- Nishimura, I., Yang, Y., and Lu, B. (2004). PAR-1 kinase plays an initiator role in a temporally ordered phosphorylation process that confers tau toxicity in *Drosophila*. *Cell* 116:671–682.
- Nollen, E.A., Garcia, S.M., van Haften, G., Kim, S., Chavez, A., Morimoto, R.I., and Plasterk, R.H. (2004). Genome-wide RNA interference screen identifies previously undescribed regulators of polyglutamine aggregation. *Proc. Natl. Acad. Sci. USA* 101:6403–6408.
- Ordway, J.M., Tallaksen-Greene, S., Gutekunst, C.A., Bernstein, E.M., Cearley, J.A., Wiener, H.W., Dure, L.S.t., Lindsey, R., Hersch, S.M., Jope, R.S., Albin, R.L., and Detloff, P.J. (1997). Ectopically expressed CAG repeats cause intranuclear inclusions and a progressive late onset neurological phenotype in the mouse. *Cell* 91:753–763.
- Outeiro, T.F., and Lindquist, S. (2003). Yeast cells provide insight into alpha-synuclein biology and pathobiology. *Science* 302:1772–1775.
- Parker, J.A., Connolly, J.B., Wellington, C., Hayden, M., Dausset, J., and Neri, C. (2001). Expanded polyglutamines in *Caenorhabditis elegans* cause axonal abnormalities and severe dysfunction of PLM mechanosensory neurons without cell death. *Proc. Natl. Acad. Sci. USA* 98:13318–13323.
- Parsell, D.A., Taulien, J., and Lindquist, S. (1993). The role of heat-shock proteins in thermotolerance. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 339:279–285; discussion 285–276.
- Perutz, M.F. (1999). Glutamine repeats and neurodegenerative diseases: molecular aspects. *Trends Biochem. Sci.* 24:58–63.

- Perutz, M.F., Johnson, T., Suzuki, M., and Finch, J.T. (1994). Glutamine repeats as polar zippers: their possible role in inherited neurodegenerative diseases. *Proc. Natl. Acad. Sci. USA* 91:5355–5358.
- Pesah, Y., Pham, T., Burgess, H., Middlebrooks, B., Verstreken, P., Zhou, Y., Harding, M., Bellen, H., and Mardon, G. (2004). *Drosophila* parkin mutants have decreased mass and cell size and increased sensitivity to oxygen radical stress. *Development* 131:2183–2194.
- Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E.S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W.G., Lazzarini, A.M., Duvoisin, R.C., Di Iorio, G., Golbe, L.I., and Nussbaum, R.L. (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 276:2045–2047.
- Price, D.L., Tanzi, R.E., Borchelt, D.R., and Sisodia, S.S. (1998). Alzheimer's disease: genetic studies and transgenic models. *Annu. Rev. Genet.* 32:461–493.
- Przedborski, S., Jackson-Lewis, V., Djaldetti, R., Liberatore, G., Vila, M., Vukosavic, S., and Almer, G. (2000). The parkinsonian toxin MPTP: Action and mechanism. *Restor. Neurol. Neurosci.* 16:135–142.
- Puls, I., Jonnakuty, C., LaMonte, B.H., Holzbaur, E.L., Tokito, M., Mann, E., Floeter, M.K., Bidus, K., Drayna, D., Oh, S.J., Brown, R.H., Jr., Ludlow, C.L., and Fischbeck, K.H. (2003). Mutant dynactin in motor neuron disease. *Nat. Genet.* 33:455–456.
- Ravikumar, B., Duden, R., and Rubinshtein, D.C. (2002). Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. *Hum. Mol. Genet.* 11:1107–1117.
- Ravikumar, B., Vacher, C., Berger, Z., Davies, J.E., Luo, S., Oroz, L.G., Scaravilli, F., Easton, D.F., Duden, R., O'Kane, C.J., and Rubinshtein, D.C. (2004). Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat. Genet.* 36:585–595.
- Ross, C.A., Margolis, R.L., Rosenblatt, A., Ranen, N.G., Becher, M.W., and Aylward, E. (1997). Huntington disease and the related disorder, dentatorubral-pallidoluysian atrophy (DRPLA). *Medicine* 76:305–338.
- Ross, C.A., Wood, J.D., Schilling, G., Peters, M.F., Nucifora, F.C., Jr., Cooper, J.K., Sharp, A.H., Margolis, R.L., and Borchelt, D.R. (1999). Polyglutamine pathogenesis. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 354:1005–1011.
- Rubin, G.M., Yandell, M.D., Wortman, J.R., Gabor Miklos, G.L., Nelson, C.R., Hariharan, I.K., Fortini, M.E., Li, P.W., Apweiler, R., Fleischmann, W., Cherry, J.M., Henikoff, S., Skupski, M.P., Misra, S., Ashburner, M., Birney, E., Boguski, M.S., Brody, T., Brokstein, P., Celniker, S.E., Chervitz, S.A., Coates, D., Cravchik, A., Gabrielian, A., Galle, R.F., Gelbart, W.M., George, R.A., Goldstein, L.S., Gong, F., Guan, P., Harris, N.L., Hay, B.A., Hoskins, R.A., Li, J., Li, Z., Hynes, R.O., Jones, S.J., Kuehl, P.M., Lemaitre, B., Littleton, J.T., Morrison, D.K., Mungall, C., O'Farrell, P.H., Pickeral, O.K., Shue, C., Vossahl, L.B., Zhang, J., Zhao, Q., Zheng, X.H., and Lewis, S. (2000). Comparative genomics of the eukaryotes. *Science* 287:2204–2215.
- Satterfield, T.F., Jackson, S.M., and Pallanck, L.J. (2002). A *Drosophila* homolog of the polyglutamine disease gene SCA2 is a dosage-sensitive regulator of actin filament formation. *Genetics* 162:1687–1702.
- Scherzer, C.R., Jensen, R.V., Gullans, S.R., and Feany, M.B. (2003). Gene expression changes presage neurodegeneration in a *Drosophila* model of Parkinson's disease. *Hum. Mol. Genet.* 12:2457–2466.
- Schlossmacher, M.G., Frosch, M.P., Gai, W.P., Medina, M., Sharma, N., Forno, L., Ochiishi, T., Shimura, H., Sharon, R., Hattori, N., Langston, J.W., Mizuno, Y., Hyman, B.T., Selkoe, D.J., and Kosik, K.S. (2002). Parkin localizes to the Lewy bodies of Parkinson disease and dementia with Lewy bodies. *Am. J. Pathol.* 160:1655–1667.
- Sherer, T.B., Kim, J.H., Betarbet, R., and Greenamyre, J.T. (2003). Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and alpha-synuclein aggregation. *Exp. Neurol.* 179:9–16.
- Shimura, H., Schlossmacher, M.G., Hattori, N., Frosch, M.P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, K.S., and Selkoe, D.J. (2001). Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. *Science* 293:263–269.
- Shulman, J.M., and Feany, M.B. (2003). Genetic modifiers of tauopathy in *Drosophila*. *Genetics* 165:1233–1242.
- Sittler, A., Lurz, R., Lueder, G., Priller, J., Lehrach, H., Hayer-Hartl, M.K., Hartl, F.U., and Wanker, E.E. (2001). Geldanamycin activates a heat shock response and inhibits huntingtin aggregation in a cell culture model of Huntington's disease. *Hum. Mol. Genet.* 10:1307–1315.
- Sperber, B.R., Leight, S., Goedert, M., and Lee, V.M. (1995). Glycogen synthase kinase-3 beta phosphorylates tau protein at multiple sites in intact cells. *Neurosci. Lett.* 197:149–153.
- Steffan, J.S., Bodai, L., Pallos, J., Poelman, M., McCampbell, A., Apostol, B.L., Kazantsev, A., Schmidt, E., Zhu, Y.Z., Greenwald, M., Kurokawa, R., Housman, D.E., Jackson, G.R., Marsh, J.L., and Thompson, L.M. (2001). Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in *Drosophila*. *Nature* 413:739–743.
- Steffan, J.S., Agrawal, N., Pallos, J., Rockabrand, E., Trotman, L.C., Slepko, N., Illes, K., Lukacovich, T., Zhu, Y.Z., Cattaneo, E., Pandolfi, P.P., Thompson, L.M., and Marsh, J.L. (2004). SUMO modification of Huntingtin and Huntington's disease pathology. *Science* 304:100–104.

- Szebenyi, G., Morfini, G.A., Babcock, A., Gould, M., Selkoe, K., Stenoien, D.L., Young, M., Faber, P.W., MacDonald, M.E., McPhaul, M.J., and Brady, S.T. (2003). Neuropathogenic forms of huntingtin and androgen receptor inhibit fast axonal transport. *Neuron* 40:41–52.
- Takahashi, M., Kanuka, H., Fujiwara, H., Koyama, A., Hasegawa, M., Miura, M., and Iwatsubo, T. (2003). Phosphorylation of alpha-synuclein characteristic of synucleinopathy lesions is recapitulated in alpha-synuclein transgenic *Drosophila*. *Neurosci. Lett.* 336:155–158.
- Takeyama, K., Ito, S., Yamamoto, A., Tanimoto, H., Furutani, T., Kanuka, H., Miura, M., Tabata, T., and Kato, S. (2002). Androgen-dependent neurodegeneration by polyglutamine-expanded human androgen receptor in *Drosophila*. *Neuron* 35:855–864.
- Thiruchelvam, M., Brockel, B.J., Richfield, E.K., Baggs, R.B., and Cory-Slechta, D.A. (2000). Potentiated and preferential effects of combined paraquat and maneb on nigrostriatal dopamine systems: environmental risk factors for Parkinson's disease? *Brain Res.* 873:225–234.
- Torroja, L., Packard, M., Gorczyca, M., White, K., and Budnik, V. (1999). The *Drosophila* beta-amyloid precursor protein homolog promotes synapse differentiation at the neuromuscular junction. *J. Neurosci.* 19:7793–7803.
- Warrick, J.M., Chan, H.Y., Gray-Board, G.L., Chai, Y., Paulson, H.L., and Bonini, N.M. (1999). Suppression of polyglutamine-mediated neurodegeneration in *Drosophila* by the molecular chaperone HSP70. *Nat. Genet.* 23:425–428.
- Warrick, J.M., Paulson, H.L., Gray-Board, G.L., Bui, Q.T., Fischbeck, K.H., Pittman, R.N., and Bonini, N.M. (1998). Expanded polyglutamine protein forms nuclear inclusions and causes neural degeneration in *Drosophila*. *Cell* 93:939–949.
- Webb, J.L., Ravikumar, B., Atkins, J., Skepper, J.N., and Rubinsztein, D.C. (2003). Alpha-Synuclein is degraded by both autophagy and the proteasome. *J. Biol. Chem.* 278:25009–25013.
- Willingham, S., Outeiro, T.F., DeVit, M.J., Lindquist, S.L., and Muchowski, P.J. (2003). Yeast genes that enhance the toxicity of a mutant huntingtin fragment or alpha-synuclein. *Science* 302:1769–1772.
- Wilson, C.A., Doms, R.W., and Lee, V.M. (2003). Distinct presenilin-dependent and presenilin-independent gamma-secretases are responsible for total cellular Abeta production. *J. Neurosci. Res.* 74:361–369.
- Wittmann, C.W., Wszolek, M.F., Shulman, J.M., Salvaterra, P.M., Lewis, J., Hutton, M., and Feany, M.B. (2001). Tauopathy in *Drosophila*: neurodegeneration without neurofibrillary tangles. *Science* 293:711–714.
- Wu, Y.R., Wang, C.K., Chen, C.M., Hsu, Y., Lin, S.J., Lin, Y.Y., Fung, H.C., Chang, K.H., and Lee-Chen, G.J. (2004). Analysis of heat-shock protein 70 gene polymorphisms and the risk of Parkinson's disease. *Hum. Genet.* 114:236–241.
- Yagi, Y., Tomita, S., Nakamura, M., and Suzuki, T. (2000). Overexpression of human amyloid precursor protein in *Drosophila*. *Mol. Cell Biol. Res. Commun.* 4:43–49.
- Yang, Y., Nishimura, I., Imai, Y., Takahashi, R., and Lu, B. (2003). Parkin suppresses dopaminergic neuron-selective neurotoxicity induced by Pael-R in *Drosophila*. *Neuron* 37:911–924.
- Ye, Y., and Fortini, M.E. (1999). Apoptotic activities of wild-type and Alzheimer's disease-related mutant presenilins in *Drosophila melanogaster*. *J. Cell Biol.* 146:1351–1364.
- Yue, S., Serra, H.G., Zoghbi, H.Y., and Orr, H.T. (2001). The spinocerebellar ataxia type 1 protein, ataxin-1, has RNA-binding activity that is inversely affected by the length of its polyglutamine tract. *Hum. Mol. Genet.* 10:25–30.
- Zhang, Y.Q., Bailey, A.M., Matthies, H.J., Renden, R.B., Smith, M.A., Speese, S.D., Rubin, G.M., and Broadie, K. (2001). *Drosophila* fragile X-related gene regulates the MAPIB homolog Futsch to control synaptic structure and function. *Cell* 107:591–603.
- Zoghbi, H.Y., and Orr, H.T. (2000). Glutamine repeats and neurodegeneration. *Annu. Rev. Neurosci.* 23:217–247.
- Zou, J., Guo, Y., Guettouche, T., Smith, D.F., and Voellmy, R. (1998). Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell* 94:471–480.

Genetically Engineered Mouse Models of Neurodegenerative Disorders

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1. Abstract

Considerable advances have been made in the past years in developing novel experimental models of neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), Fronto-Temporal Dementias (FTD), Amyotrophic Lateral Sclerosis (ALS), and trinucleotide repeat disorders (TNRDs). The main postulate of several of the genetically modified murine models of neurodegenerative disorders is that a single molecular alteration might trigger a cascade of events that eventually will result in the full spectrum of the clinicopathological alterations observed in human disease. Therefore, overexpression of mutant proteins in transgenic (tg) mice might mimic some aspects associated with the gain of a toxic function, while targeted deletion of selected genes might mimic aspects associated with loss of a trophic or protective function. To date, several genes have been identified to be associated with familial forms of AD, PD, FTD, ALS, and TNRDs. Single or combined tg and knockout models targeting most of these genes have been developed that recapitulate one or several aspects of each disorder. In these models, abnormal accumulation and misfolding (toxic conversion) of endogenous proteins is being extensively explored as a key pathogenic event leading to neurodegeneration. Thus, the main focus of this chapter is to provide a perspective as to the efforts in developing genetically engineered models of the most common neurodegenerative disorders. Further development and investigation of animal models of these diseases holds the promise of better understanding their pathogenesis and discovering and testing new treatments.

2. Introduction

Progress in the early detection and diagnosis of age-associated neurological conditions resulting in neurodegeneration such as Alzheimer's disease (AD), Parkinson's disease (PD), Fronto-Temporal Dementias (FTD), Amyotrophic Lateral Sclerosis (ALS), trinucleotide repeat disorders (TNRDs), Creutzfeld-Jacobs disease (CJD) and others has dramatically advanced our understanding of the pathogenesis of dementia and movement disorders, but have also called attention to the epidemic dimension of the problem. To date, most of these diseases are incurable, and pose a serious challenge to modern society because of their devastating effects on the individual, their family, and the health care system. Development of animal models of these diseases holds the promise of better understanding their pathogenesis and discovering and testing new treatments (Gotz *et al.*, 2004).

The key neuropathological feature common to these disorders that drives their unique neurological presentation is the neurodegeneration of selective neuronal populations and circuitries (Hof

and Morrison, 1994). Neurodegeneration has been classically defined as the loss of specific neuronal populations accompanied by gliosis. However, it is now clear that neuronal cell death might occur later in the progression of the disease and that damage to the synaptodendritic apparatus might be one of the earliest pathological alterations (Masliah and Terry, 1993, 1994; Masliah, 1998, 2001; Honer, 2003; Scheff and Price, 2003). This is accompanied by the abnormal accumulation of neuronal proteins that are manifested neuropathologically as extracellular lesions [e.g., plaques, cerebral amyloid angiopathy (CAA)] or intracellular inclusions [e.g., tangles, Lewy bodies (LBs), Marinesco bodies]. Thus, when developing ideal genetically engineered experimental murine models, it is expected that overexpression or deletion of specific neural proteins will result in selective damage to neuronal circuitries that leads to cognitive and/or motor impairment accompanied by the formation of the diagnostic lesions of the diseases. The great majority of these neurodegenerative disorders can be divided into familial and sporadic forms. Familial forms (dominant or recessive) are often associated with mutations or multiplications of genes, while the more common sporadic variants are probably the combined effect of environmental neurotoxic factors and genetic polymorphisms.

Considerable advances have been made in the past years in developing novel experimental models of neurodegenerative disorders (Ahmad-Annuar *et al.*, 2003). This dramatic progress has, in part, been driven by the identification of genetic mutations associated with familial forms of these conditions and gene polymorphisms associated with the more common sporadic variants of these diseases. Furthermore, the implementation and adaptation of molecular techniques to direct the expression of the transgenes in specific cellular populations in the brain has had a profound effect in the development of animal models for neurodegenerative disorders (Mucke *et al.*, 1991). To date, several genes have been identified to be associated with familial forms of AD, PD, FTD, ALS, TNRDs, and CJD. Single or combined transgenic (tg) and knockout (KO) models targeting most of these genes in the rat, mouse (Heintz and Zoghbi, 2000), *Drosophila* (Shulman *et al.*, 2003), and *Caenorhabditis elegans* (Driscoll and Gerstbrein, 2003) have been developed (Aguzzi *et al.*, 1996; Aguzzi and Raeber, 1998). However, it is important to emphasize that although each of these models recapitulates one or various aspects of the disease, none of them completely reproduces all the clinicopathological alterations of each disease. This is in part because of the more complex nature of the neurodegenerative process and inherited heterogeneity of the disease in humans, the compensatory effects of multiple genes, and the differences in opinion in the classification of these diseases because of the advances in the understanding of their pathogenesis. For example, the clinical and pathological features of AD and PD often overlap (Galasko *et al.*, 1994), and cytoskeletal pathology involving microtubule-associated proteins and neurofilaments (NFs) is common to several neurodegenerative disorders such as AD, PD, ALS, FTD, and progressive supranuclear palsy (PSP) (Trojanowski *et al.*, 1993). Because of the complex presentation of these disorders, efforts have been made in developing new classifications based on the key genes affected and abnormal proteins accumulating. This has led to the concept of tauopathies, synucleopathies, amyloidosis, prion disease, and others to refer to FTD, Lewy body disease (LBD)/PD, AD, and CJD and their variants, respectively (Hardy and Gwinn-Hardy, 1998; Litvan, 1999).

Abnormal accumulation and misfolding (toxic conversion) of neuronal proteins that localize to the synapses is being extensively explored as a key pathogenic event leading to neurodegeneration in PD, LBD, AD, and other neurological disorders (Koo *et al.*, 1999; Ramassamy *et al.*, 1999; Ferrigno and Silver, 2000) (Figure 13.2-1). In PD and related conditions such as LBD, abnormal accumulation of α -synuclein occurs in neuronal cell bodies, axons, and synapses (Spillantini *et al.*, 1997; Irizarry *et al.*, 1998; Takeda *et al.*, 1998), while in AD, misfolded amyloid- β peptide 1–42 ($A\beta_{1-42}$) a proteolytic product of amyloid precursor protein (APP) metabolism accumulates in the

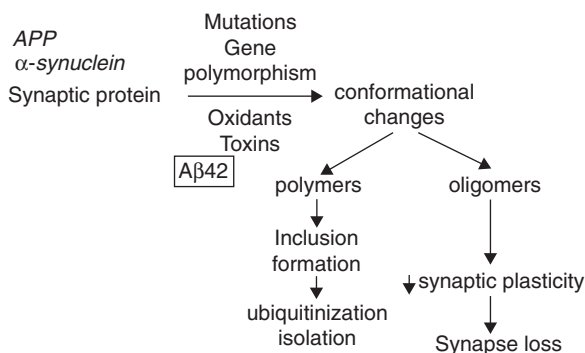


Figure 13.2-1. Diagrammatic representation of the potential mechanisms through which protein misfolding leads to synapse damage and neurodegeneration

neuronal endoplasmic reticulum (ER) and extracellularly (Selkoe *et al.*, 1996; Trojanowski and Lee, 2000; Walsh *et al.*, 2000). In contrast, in TNRDs such as Huntington's disease (HD), accumulation of misfolded proteins occurs in the neuronal cytosol and nuclei (Cummings and Zoghbi, 2000; Muchowski, 2002). The key pathogenic event triggering synaptic loss and selective neuronal cell death in these disorders is not yet completely clear (Masliah, 2000, 2001); however, recent studies suggest that nerve damage might result from the conversion of normally nontoxic monomers (and small oligomers) to toxic oligomers and protofibrils (Volles *et al.*, 2001; Volles and Lansbury, 2002), whereas larger polymers and fibers that often constitute the intracellular inclusions and extracellular lesions might not be as toxic (Lansbury, 1999) (Figure 13.2-1).

The main postulate of several of the genetically modified murine models of neurodegenerative disorders is that a single molecular alteration might trigger a cascade of events that eventually will result in the full spectrum of the clinicopathological alterations observed in human disease. Therefore, overexpression of mutant proteins in tg mice might mimic some aspects associated with the gain of a toxic function, while targeted deletion of selected genes might mimic aspects associated with loss of a trophic or protective function. Furthermore, and as indicated previously, overexpression of a single molecule associated with familial variants of a neurodegenerative disorder might not necessarily result in the development of all the characteristics of the disease, but in an increased susceptibility to developing the disease process provided that the appropriate pathogenic factors are present.

In this context, the main objectives of developing genetically modified murine models of neurodegenerative disorders are: (1) to define the time course of molecular events associated with the development of the disease, (2) to study the progression of the neurodegenerative process, (3) to determine the individual pathogenic role of specific molecules or specific mutations *in vivo*, (4) to study novel mechanisms involved in the disease, and (5) to develop models for the testing of novel therapies.

Thus, the main focus of this chapter is to provide a perspective as to the efforts in developing genetically engineered models of the most common neurodegenerative disorders. Several reviews have been recently published on this subject (Beal, 2001; Gotz, 2001; Shibata, 2001; Betarbet *et al.*, 2002; Dawson *et al.*, 2002; Newbery and Abbott, 2002; Trojanowski *et al.*, 2002; Higgins and Jacobsen, 2003; Gotz *et al.*, 2004), as well as for genetically engineered models of neuroinflammatory

disorders (Campbell *et al.*, 1998) and prion diseases (Prusiner and Scott, 1997; Aguzzi and Raeber, 1998), and the reader is advised to consult them for additional information.

3. Alzheimer's Disease and Cerebrovascular Amyloidosis

3.1. Modeling the Pathogenesis of AD in Animals

Alzheimer's disease is the most common neurodegenerative disorder in the aging population, and is characterized by the progressive and irreversible deafferentation of the limbic system, association neocortex, and basal forebrain (Perry *et al.*, 1977; Hyman *et al.*, 1984; Wilcock *et al.*, 1988; Hof *et al.*, 1990; Palmer and Gershon, 1990; Masliah *et al.*, 1993), accompanied by the formation of neuritic plaques, neurofibrillary tangles (NFTs) and neuropil threads (Terry *et al.*, 1994). This neurodegenerative process is followed by reactive astrogliosis (Dickson *et al.*, 1988) and microglial cell proliferation (Rogers *et al.*, 1988; Masliah *et al.*, 1991). Therefore, designing experimental animal models of AD is a complex and difficult task, because this disease manifests with alterations at many levels ranging from molecular, subcellular, and cellular to neurophysiological and cognitive. Experimental models of AD could mimic individual or multiple alterations found in AD; however, to this date not a single model mimics all the alterations observed in AD. Most of the recently developed tg animal models of AD are based on the targeted overexpression of single or multiple mutant molecules associated with familial AD (FAD). Currently, mutations in three genes have been described, namely APP, presenilin (PS)1 and PS2 (Hutton and Hardy, 1997; Cruts and Van Broeckhoven, 1998; Rocchi *et al.*, 2003; Bertoli-Avella *et al.*, 2004; Pastor and Goate, 2004).

Although the precise mechanisms leading to neurodegeneration in AD are not completely clear, recent studies have focused on the role of APP and its products in AD pathogenesis. Amyloid precursor protein has been centrally implicated in the pathogenesis of AD (Masters *et al.*, 1985; Selkoe, 1989), because mutations within this molecule are associated with FAD (Goate *et al.*, 1991; Clark and Goate, 1993), overexpression of mutated APP in tg mice results in AD-like pathology (Games *et al.*, 1995), and APP degradation products accumulate in brains of patients with AD (Sisodia *et al.*, 1990). Furthermore, APP abnormally accumulates both in dystrophic neurites of plaques and in synaptic terminals (Cole *et al.*, 1989; Martin *et al.*, 1989; Arai *et al.*, 1991; Cras *et al.*, 1991; Masliah *et al.*, 1992; Masliah, 1995). In AD, the balance between APP isoforms is altered with a relative shift toward increase in APP770/751 versus APP695 (Rockenstein *et al.*, 1995). Therefore, because APP is centrally involved in AD pathogenesis, most of the studies involving tg animal models have focused on overexpressing APP in an attempt to reproduce aspects of AD-like pathology.

3.2. APP tg Models of AD

Recently developed tg animal models have shown that it is possible to reproduce certain aspects of AD pathology in a shorter period of time (Masliah *et al.*, 1996b; Games *et al.*, 1997; Price *et al.*, 2000) (Table 13.2-1). Of these tg models, the first one to unquestionably show the development of robust plaque pathology was the model of Games *et al.* (1995) (Table 13.2-1). This model was based on the concept that overexpression of mutant APP (or its fragments) at high levels under the control of neuron-specific promoters might result in AD-like pathology. The platelet-derived growth factor (β -chain) (PDGFR β) promoter drives an alternatively spliced human APP (hAPP) minigene (PDAPP) encoding mutated V $\rightarrow$ F hAPP695, 751, 770 (Games *et al.*, 1995; Rockenstein *et al.*, 1995). Compared to both nontransgenic (nontg) mice and humans, PDAPP tg mice showed four- to sixfold higher levels of total APP messenger RNA (mRNA), while their endogenous mouse APP mRNA levels were reduced. This resulted in a high ratio of mRNA encoding mutated hAPP versus the wild-type (wt)

Table 13.2-1. Singly-transgenic models of APP expression

Promoter	Construct	Mutation(s)	Phenotype	Reference(s)
mThy-1	hAPP751	Sw + Lon	amyloid deposits, neuritic plaques, high levels of A β 42	(Rockenstein <i>et al.</i> , 2001)
mThy-1	hAPP695	Sw, Lon	amyloid plaques and CAA, high levels of A β 40	(Moechars <i>et al.</i> , 1999)
mThy-1	hAPP751	Sw	dense amyloid plaques, high levels of A β 40	(Sturchler-Pierrat <i>et al.</i> , 1997)
mThy-1	hAPP770	Fl, D	behavioral deficits without amyloid deposits	(Kumar-Singh <i>et al.</i> , 2000)
m or hThy-1	hAPP751	Sw	dense amyloid plaques	(Andra <i>et al.</i> , 1996)
hThy-1	hAPP751	Sw, Sw + Lon	dense amyloid plaques	(Sturchler-Pierrat <i>et al.</i> , 1997)
Hamster PrP	hAPP695	Sw + Ind	early onset dense amyloid plaques and neuritic pathology, high levels of A β 42	(Chishti <i>et al.</i> , 2001)
Hamster PrP	hAPP695	Sw	amyloid deposits, high levels of A β 40	(Hsiao <i>et al.</i> , 1996)
PDGF β	hAPP695, 751, 770	Ind	diffuse and mature amyloid plaques, high levels of A β 42	(Games <i>et al.</i> , 1995)
PDGF β	hAPP695, 751, 770	Sw + Ind	synapse pathology, memory deficits, amyloid plaques, high levels of total A β	(Hsia <i>et al.</i> , 1999; Mucke <i>et al.</i> , 2000b)
NF-L	hAPP591–695	NA	A β extracellular immunoreactivity, hippocampal cell loss	(Nalbantoglu <i>et al.</i> , 1997)
PDGF β	hAPP695, 751, 770	A + Sw + Ind	faster and more extensive amyloid plaque formation	(Cheng <i>et al.</i> , 2004)

Mutations: Sw, Swedish (K670M/N671L) APPL; Lon, London (V717I) APP; Ind, Indiana (V717F) APP; Fl, Flemish (A682G) A β ; D, Dutch (E693Q) APP; A, Arctic (E22G) A β .

mouse APP (Rockenstein *et al.*, 1995), and promoted development of typical amyloid plaques, dystrophic neurites, loss of presynaptic terminals, astrogliosis, and microgliosis (Games *et al.*, 1995, 1997; Masliah *et al.*, 1996b).

The success of the PDAPP tg model is based on high levels of expression driven by the PDGF β promoter in the context of the minigene in which alternatively spliced mutant APP is generated. This construct is rather complex and difficult to stabilize, and the triple background of the mice [Swiss Webster X B6D2F1(C57Bl/6 X DBA/2)] might also play an important role. However, more recent studies have shown that it is actually possible to induce significant plaque pathology utilizing alternative constructs. Two tg mouse lines have also been shown to develop pathological features reminiscent of AD (Andra *et al.*, 1996), and the degree of pathology was dependent on expression levels and specific mutations. The construct consisted of a human or murine (m) Thy-1 promoter driving mutant APP751 (Table 13.2-1). These mice were generated in B6D2F1 X B6D2F1 embryos, and mice were bred by backcrossing with C57Bl/6. A twofold overexpression of hAPP with the Swedish double mutation (K670M/N671L) combined with the London (V717I) mutation resulted in diffuse A β deposition (**Figure 13.2-2A; see color insert**) in the neocortex and hippocampus of 18-month-old tg mice. In mice with sevenfold overexpression of hAPP harboring the Swedish mutation alone, senile plaques appeared at 6 months. In these latter mice the plaques showed astroglial reaction accompanied by Tau pathology (Andra *et al.*, 1996; Sturchler-Pierrat *et al.*, 1997) (**Figure 13.2-2C; see color insert**), but no paired-helical filaments (PHFs) or NFTs were observed. Finally,

Table 13.2-2. Doubly-transgenic models of APP expression

Cross	Phenotype	Reference(s)
APP × PS1 tg	accelerated plaque formation	(Borchelt <i>et al.</i> , 1996; Duff, 1997; Holcomb <i>et al.</i> , 1998; McGowan <i>et al.</i> , 1999; Siman <i>et al.</i> , 2000; Jankowsky <i>et al.</i> , 2001; Schmitz <i>et al.</i> , 2004)
APP × TGFβ1 tg	amyloid angiopathy	(Wyss-Coray <i>et al.</i> , 1997)
APP × APOE3, APOE4 tg	decreased amyloid deposition, modulation of AD-like synaptic and cholinergic pathology	(Holtzman <i>et al.</i> , 1999; Buttni <i>et al.</i> , 2002)
APP × APOE KO	decreased amyloid deposition	(Bales <i>et al.</i> , 1997)
APP × ACT tg	increased amyloid deposition	(Abraham <i>et al.</i> , 2000)
APP × SOD tg	decreased mortality	(Carlson <i>et al.</i> , 1997)
APP × FGF2 tg	increased mortality	(Carlson <i>et al.</i> , 1997)
APP × RAP KO	increased amyloid deposition, neuronal damage and astrogliosis	(Van Uden <i>et al.</i> , 2002)
APP × Tau tg	increased NFT formation	(Lewis <i>et al.</i> , 2001)
APP × α-synuclein tg	increased α-synuclein fibrillar inclusions and severe behavioral deficits	(Masliah <i>et al.</i> , 2001b)
APP × PS1 × Tau tg	accelerated plaque formation and tangles present, amyloid deposition precedes tangle formation	(Oddo <i>et al.</i> , 2003b; Boutajangout <i>et al.</i> , 2004)
APP × SOD KO	increased plaque formation	(Li <i>et al.</i> , 2004)

Moechars *et al.* (1999) have shown similar alterations using the hThy-1 promoter driving a mutant hAPP695 cDNA.

More recently, other models have expressed mutant hAPP under the regulatory control of either the human or mThy-1 promoter (Andra *et al.*, 1996; Sturchler-Pierrat *et al.*, 1997; Moechars *et al.*, 1999; Bornemann and Staufenbiel, 2000) or the protease-resistant prion protein (PrP) promoter (Hsiao *et al.*, 1996; Borchelt *et al.*, 1997) (Table 13.2-1). In these mice plaque formation begins at 12 months of age; however, coexpression of mutant PS1 accelerates amyloid deposition (Table 13.2-2), which begins at 4 months of age (Borchelt *et al.*, 1996, 1997; Holcomb *et al.*, 1998). Another more recently developed model, where APP is also expressed under the control of the PrP promoter, displays even earlier onset of amyloid deposition, starting at 3 months and progressing to mature plaques and neuritic pathology from 5 months of age, accompanied by high levels of Aβ₁₋₄₂ (Chishti *et al.*, 2001). Although the PrP promoter has provided several models that mimic aspects of FAD, other promoters targeting expression of APP to neurons provide alternative models demonstrating pathology that recapitulates similar and additional aspects of FAD. In this regard, we have generated lines of tg mice expressing hAPP751 cDNA containing the London and Swedish mutations under the regulatory control of the mThy-1 gene (mThy1-hAPP751) (Rockenstein *et al.*, 2001) (Table 13.2-1). In the brains of the highest (Line 41) and intermediate (Lines 16 and 11) expressers, high levels of hAPP expression were found in neurons in layers 4–5 of the neocortex, hippocampal CA1, and olfactory bulb. At as early as 3–4 months of age, Line 41 mice developed mature plaques in the frontal cortex (**Figure 13.2-2B; see color insert**), while at 5–7 months plaque formation extended to the hippocampus, thalamus, and olfactory region. Ultrastructural and double-immunolabeling analysis confirmed that most plaques were mature, and contained dystrophic neurites immunoreactive with antibodies against APP, synaptophysin, NF, and Tau. In addition, a decrease in the number of

synaptophysin-immunoreactive terminals was most prominent in the frontal cortex of mice from Line 41. Mice from Line 11 developed diffuse amyloid deposits at 11 months of age, while mice from Line 16 did not show evidence of amyloid deposition. Analysis of A β by ELISA showed that levels of A β ₁₋₄₀ were higher in mice that did not show any amyloid deposits (Line 16), while A β ₁₋₄₂ was the predominant species in tg animals from the lines showing plaque formation (Lines 41 and 11) (Rockenstein *et al.*, 2001).

Therefore, although expression of mutant hAPP under the PDGF β promoter results in the production of diffuse (**Figure 13.2-2A; see color insert**) (and some mature) plaques (Games *et al.*, 1995; Mucke *et al.*, 2000b), tg expression of mutant hAPP under the Thy-1 (Andra *et al.*, 1996) and PrP (Hsiao *et al.*, 1996; Borchelt *et al.*, 1997) promoters favors the formation of mature plaques (**Figure 13.2-2B; see color insert**) in the hippocampus and neocortex. This suggests that the differential patterns of A β deposition might be dependent on the specific neuronal populations selected by the promoter, levels of expression, and topographical distribution of the transgene. Nonetheless, it is worth noting that we observed the formation of diffuse plaques in older lower expresser mThy1-hAPP751 tg mice (Line 11), suggesting that other factors might be at play. Among them, our studies have shown that plaque-producing tg mice (Lines 11 and 41) differed from plaque nonproducing tg mice (Line 16) not only in levels of mutant hAPP expression, but also in the ratio and overall levels of A β ₁₋₄₀ and A β ₁₋₄₂. Consistent with this, in FAD and Down syndrome, production of high levels of A β ₁₋₄₂ results in early plaque formation (Citron *et al.*, 1997). This suggests that age of onset and plaque type might be dependent on the levels of A β ₁₋₄₀ and A β ₁₋₄₂ production (Rockenstein *et al.*, 2001). For a detailed review of these and additional tg models of neurodegenerative disease, please visit the Alzheimer's Forum Web site at: <http://www.alzforum.org/res/com/tra>.

3.3. Behavioral Deficits and Neurodegeneration in APP tg Models of AD

The relationship among the patterns of amyloid deposition, neurodegeneration, and behavioral deficits in the tg models of AD is complex (Morgan, 2003). One important finding common to several of these APP tg models is that there is no obvious neuronal dropout in early stages (Irizarry *et al.*, 1997a, 1997b). In fact, the earliest neuronal pathology before amyloid deposition is the loss of synapses and dendrites in the limbic system and neocortex (Hsia *et al.*, 1999; Mucke *et al.*, 2000b) (**Figure 13.2-2D and E; see color insert**). This is accompanied by neurophysiological deficits and alterations in long-term potentiation (LTP) and field potential (Chapman *et al.*, 1999; Hsia *et al.*, 1999). Because the synaptic damage in these mice correlates better with the levels of soluble A β ₁₋₄₂ than with plaques, it has been proposed that neurodegeneration might be associated more with the neurotoxic effect of A β oligomers than with fibrillar amyloid (Mucke *et al.*, 2000b). Furthermore, behavioral deficits in the water maze are more closely associated with synaptic damage and neurodegeneration than with plaque formation (Berger-Sweeney *et al.*, 1999; Masliah and Rockenstein, 2000). These results indicate that similarly to patients with preclinical AD, in tg mice the earliest manifestation of the neurodegenerative process is the loss of synaptic input. This, in turn, could lead via anterograde or retrograde mechanisms to neuronal dysfunction and loss. A neuronal population selectively sensitive to the effects of oligomers and disruption of the synaptic circuitries in the hippocampus is the calbindin-immunoreactive granular cells in the dentate gyrus (Palop *et al.*, 2003). The loss of this calcium-binding molecule in granular cells is a strong predictor of the functional alterations in this circuitry, and strongly correlates with the learning and memory deficits in the water maze. Overall neuronal damage and disruption of the synaptic circuitries is a more prominent feature in the tg APP models rather than frank neuronal loss, indicating that other factors might be at play, including an increased resistance to amyloid toxicity in murine models. Other events that might also

be involved in amyloidogenesis include the contribution of the neuroinflammatory response to neurodegeneration. To investigate this possibility and to assess the importance of complement in the pathogenesis of neurodegeneration in AD models, recent tg models have been developed where APP tg mice were crossed with tg mice to inhibit C3 complement activation (Wyss-Coray *et al.*, 2002) (Table 13.2-2). In addition to the overexpression of hAPP, the soluble complement receptor-related protein y (sCrry), a complement inhibitor, is also expressed in the brains of these mice. This down-regulation of the neuroinflammatory process via the inhibition of complement activation led to a two- to threefold increase in A β deposition and neurodegeneration (**Figure 13.2-2F**; see color insert) compared to age-matched APP tg controls. This suggests that complement activation products play an important role in reducing the accumulation or promoting the clearance of amyloid and degenerating neurons in models of AD.

Other recently developed models have also focused on defining the effects of APP and its products on functional markers, including behavioral performance and LTP. These studies have shown that overexpression of the C-terminal APP fragment C100 under the control of NF promoter results in amyloid-like production and electrophysiological alterations (Nalbantoglu *et al.*, 1997) (Table 13.2-1). Compared to the PDAPP model, these tg mice display mild AD-like pathology. In this model, high intraneuronal expression of the murine A β peptide under the control of the low molecular weight NF (NF-L) promoter induced premature death with widespread DNA fragmentation and reactive gliosis in the central nervous system (CNS). Targeting of the same construct to the extracellular compartment in the brain by adding a signal peptide of neural cell adhesion molecule to the construct did not result in any phenotype (LaFerla *et al.*, 1995), suggesting that the presence of A β inside the neurons is particularly detrimental (Table 13.2-1). Other features of AD pathology (plaques, tangles, and dystrophic neurites) were absent, and it is unclear to which cellular compartment the transgene-derived A β peptide localizes (Aguzzi *et al.*, 1996). Several other tg mice overexpressing mutant APP, A β , or the C100 region under the control of several different promoters have been generated; however, none of them show significant neuropathological features mimicking AD. Although these, as well as the other APP tg animal models, have been shown to be of significant interest, the basic principle for their success rests in the ability of overexpressing high levels of mutant APP, which in a way is a rather nonphysiological event and, with the exception of Down syndrome, in sporadic AD there is no evidence for upregulation in APP expression, but rather a shift in the ratio between APP770, 751 to APP695. In this regard, a recent study (van Leeuwen *et al.*, 1998) has shown frame shift mutations in APP and ubiquitin genes of AD. It is conceivable that future tg models might utilize this mechanism in an attempt to reflect the more common sporadic forms of the disease.

3.4. Crosses of APP tgs with Other Lines

Current development in this field has been oriented toward defining the role of additional molecular events involved in amyloidogenesis and neurodegeneration in AD. For this purpose, crosses of APP tg mice with a variety of other tg mice overexpressing proteins that interact with the development and processing of amyloid have been established (Table 13.2-2). For example, mice produced by crossing PDAPP tg with human α 1-antichymotrypsin (hACT) tg mice show increased amyloid load without a modification in age of onset (Mucke *et al.*, 2000a). Transgenic mice overexpressing mutated hAPP695 under the control of the PrP promoter showed behavioral deficits at 9–10 months of age, followed by formation of mature plaques at 12–18 months of age (Hsiao *et al.*, 1996). Moreover, although transforming growth factor β 1 (TGF β 1) promotes enhanced amyloid deposition in the blood vessels (Wyss-Coray *et al.*, 2001) in the PDAPP tg mice, apolipoprotein E (apoE) delays amyloid deposition (Holtzman *et al.*, 1999). In crosses between APP tg mice and apoE-KO mice, plaque formation and amyloid deposition is greatly reduced (Bales *et al.*, 1997). Surprisingly, crosses

of APP tg mice with apoE 3 or 4 tg mice also result in decreased plaque formation rather than accelerated amyloid deposition (Holtzman *et al.*, 1999). Although apoE delays amyloid deposition, one of its receptors, the low-density lipoprotein receptor-related protein (LRP), mediates the degradation of amyloid (Van Uden *et al.*, 2000). In crosses between hAPP tg mice and a model of decreased LRP expression, receptor-associated protein (RAP)-deficient mice, levels of amyloid deposition increased significantly and animals displayed increased neuronal damage and astrogliosis (Van Uden *et al.*, 2002). These findings support the role of LRP in A β clearance and its potential neuroprotective effects against amyloid toxicity. Other factors may also be at play in the pathogenesis of neurodegeneration in AD models, as shown in crosses between APP tg and SOD1 tg mice that are protective, suggesting involvement of oxidative damage in premature death (Table 13.2-2). In contrast, overexpression of the fibroblast growth factor 2 (FGF2) transgene enhanced the lethal effects of APP (Table 13.2-2) (Carlson *et al.*, 1997). This differential survival does not appear to reflect genetic differences in APP processing, but rather host responses to APP or its derivatives.

Several studies have shown that both TGF β 1 (Wyss-Coray *et al.*, 1997) and PS (Borchelt *et al.*, 1997) accelerate amyloid production and deposition. In the case of TGF β 1, these deposits are found around blood vessels, and this model might be an interesting paradigm to study amyloid angiopathy, which is also a feature of AD. Previous reports (Borchelt *et al.*, 1997), have demonstrated that tg animals coexpressing a FAD-linked human PS1 variant (A246E) and a chimeric m/hAPP harboring mutations linked to Swedish FAD develop numerous amyloid deposits much earlier than age-matched mice expressing APP (Swedish) and wt human PS1 or APP (Swedish) alone. Other APPxPS1 crosses (Duff *et al.*, 1996; McGowan *et al.*, 1999) exhibit similar characteristics, and taken together, these results provide evidence for the view that one pathogenic mechanism by which FAD-linked mutant PS1 causes AD is by accelerating the rate of A β deposition in the brain (Table 13.2-2). Similar results have also been reported in the mutant APP (K670N,M671L) tg line (Holcomb *et al.*, 1998), Tg2576, which shows significantly elevated A β levels at an early age and, by 9–12 months, extracellular AD-type A β deposits in both the cortex and hippocampus. Mutant PS1 tg mice do not show abnormal pathology, but display subtly elevated levels of the highly amyloidogenic 42- or 43-amino acid peptide A β ₁₋₄₂ (Duff *et al.*, 1996). Furthermore, another recent study describes an APPxPS1 cross (Schmitz *et al.*, 2004) that indicates that this combination is a key factor in demonstrating not only amyloid deposition, but the neuronal loss prevalent in AD.

3.5. Models of Cerebrovascular Amyloidosis

Despite the progress with APPxPS1 tg mice, the factors that initiate or promote deposition of A β peptide are not completely known. TGF β 1 plays a central role in the response of the brain to injury; increased TGF β 1 has been found in the CNS of patients with AD, and it induces A β deposition in cerebral blood vessels (**Figure 13.2-2G; see color insert**) and meninges of aged tg mice overexpressing this cytokine from astrocytes (Wyss-Coray *et al.*, 1997). Coexpression of TGF β 1 in tg mice overexpressing APP (PDAPP transgene), which develop AD-like pathology, accelerate the deposition of A β peptide. More TGF β 1 mRNA was present in postmortem brain tissue of AD patients than in controls, and the levels correlated strongly with A β deposition in the damaged cerebral blood vessels of patients with CAA (Wyss-Coray *et al.*, 1997). These results indicate that high levels of TGF β 1 may initiate (or promote) amyloidogenesis in AD and in experimental models of AD, and so may be considered as a risk factor.

In CAA, amyloid deposits build up in the walls of leptomeningeal and cortical arteries, arterioles, and sometimes in capillaries and veins (Mandybur, 1986; Ghiso and Frangione, 2001). The most common form of CAA is caused by mutations in APP. Hereditary cerebral hemorrhage with amyloidosis (HCHWA) is associated with a mutation within the A β domain of APP. Furthermore, the apoE

genotype is known to be a risk factor for CAA (Fryer *et al.*, 2003). Because both AD and CAA share common molecules that contribute to the pathogenesis of these diseases, several APP singly-tg models have also been advantageous in elucidating the mechanisms of CAA. These models have been shown to develop vascular amyloid deposition in addition to the characteristic parenchymal lesions mimicking amyloid plaques (Herzig *et al.*, 2004). This is of importance because in AD patients amyloid angiopathy is also a significant feature. In these patients and tg animal models $A\beta_{1-42}$ is more abundant in the plaques and $A\beta_{1-40}$ is the predominant form in the cerebrovascular amyloid deposits (Ozawa *et al.*, 2002; Nicoll *et al.*, 2004). Interestingly, overexpression of APP targeted to brain vascular endothelial cells in a tg model (Jahroudi *et al.*, 2003) provides insight into the contribution of vascular cells to the pathogenesis of cerebrovascular disease. In this model, hAPP was expressed under the regulation of the Von Willebrand factor promoter, a gene promoter fragment that targets gene expression specifically to brain vascular endothelial cells. This approach may be useful to help elucidate the precise mechanisms that confer CAA, and how these interact with and differ from mechanisms of AD pathogenesis.

Recent studies have shown that while mutations flanking the β and γ secretase sites of APP are associated with increased $A\beta_{1-42}$ production and early onset FAD, mutations within the $A\beta$ domain result in predominantly cerebrovascular pathology, in the absence of mature plaque formation (Hardy, 1997; Selkoe, 1999; Haass and Steiner, 2001; Herzig *et al.*, 2004). For instance, patients with the Dutch variant of the HCHWA autosomal form of CAA display a point mutation in affecting residue 22 of $A\beta$ (E693Q) (Herzig *et al.*, 2004). CAA is typically found in leptomeningial vessels and parenchymal vessels leading to hemorrhages. Consistent with this, in tg mice, overexpression of mutant APP E693Q under the neuronal Thy-1 promoter results in extensive CAA with hemorrhages (Herzig *et al.*, 2004), and in these mice $A\beta_{1-40}$ is the predominant form in the vessels. Crossing the APP Dutch mice with mutant PS1 tg animals results in increased generation of $A\beta_{1-42}$ and redistribution of $A\beta$ from the vessels to the parenchyma (Herzig *et al.*, 2004).

3.6. Neurofibrillary Pathology in tg Models of AD

Although most of the APP tg models develop several aspects of AD including synaptic dysfunction, memory deficits, and amyloid deposition, modeling neurofibrillary pathology has been more difficult. The reason for this is unclear; however, some mutant APP tg models develop mild neurofibrillary pathology, but no PHFs or NFTs (Masliah *et al.*, 2001a) (for review, see Higgins and Jacobsen, 2003). Abnormally phosphorylated Tau is a major component of NFTs, and recent studies have linked Tau mutations to FTD with parkinsonism linked to chromosome 17 (FTDP-17) (Hutton *et al.*, 1998; Poorkaj *et al.*, 1998), suggesting that dysfunction of Tau, as well as formation of $A\beta$, can lead to neurodegeneration and dementia. Newly established tg mice overexpressing either mutant forms of Tau (e.g., P301L) alone or in combination with APP have been developed (Lewis *et al.*, 2001). Neurofibrillary tangles composed of a highly-phosphorylated form of Tau are a characteristic neuropathological lesion of AD (Kosik, 1990; Brion, 1998). The tangle component of AD pathology is still missing from all current tg models, and it appears that simply overexpressing Tau in neurons (a successful strategy in APP tg mice) might not be responsible for NFT development (Boutajangout *et al.*, 2002). Although studies in tg mice expressing hTau isoforms and in animals doubly-tg for Tau and mutant PS1 have shown a somatodendritic accumulation of phosphorylated Tau proteins (similar to the pretangle stage in AD), no NFTs were found (Brion *et al.*, 2001). More recent studies in tg mice expressing hTau in central neurons showed progressive degeneration of nerves and muscles; thus, these animals cannot be tested in the Morris water maze and die prematurely (Hutton *et al.*, 2001). In tg mice overexpressing the P301L Tau mutation, Tau filament formation is accelerated (Lewis *et*

al., 2000). The selection of the P301L Tau mutation is based on studies showing that mice expressing mutant Tau develop motor and behavioral disturbances (Lewis *et al.*, 2000). In other currently available models, crossing mutant Tau tg with mutant APP tg mice resulted in the development of amyloid plaques associated with only marginal (nonlimbic) neuronal damage with some NFT pathology (Lewis *et al.*, 2001), suggesting that introduction of human disease-associated genes directly into mouse brains might provide a more comprehensive model of neurodegeneration. Further supporting this rationale, in these tg mice, A β ₁₋₄₂ accelerates and promotes Tau fibrillization and tangle formation (Gotz *et al.*, 2001a; Lewis *et al.*, 2001). Furthermore, two triple tg models have recently been developed: one expressing mutant APP, PS1, and Tau (Oddo *et al.*, 2003a, 2003b), and the other expressing mutant APP and PS1 and wt human Tau (Boutajangout *et al.*, 2004) (Table 13.2-2). These models demonstrate the progression of A β deposition and Tau pathology in the brain: although A β deposition begins in the cortex and progresses to the hippocampus, Tau pathology initially affects the hippocampus and progresses to the cortex with aging (Oddo *et al.*, 2003a). Moreover, A β deposition develops prior to tangle pathology (Boutajangout *et al.*, 2004). Taken together, these triple tg models combine several of the core pathologies of AD, and may be useful in the development of potential therapies for the treatment of AD.

4. Fronto-temporal Dementias and Tauopathies

4.1. Introduction to the Pathogenesis of Tauopathies in Animal Models

The term tauopathies has been recently adopted to refer to a heterogeneous group of neurodegenerative disorders characterized by the progressive accumulation of filamentous inclusions in selective neuronal populations and glial cells composed of the cytoskeletal protein Tau (Dickson *et al.*, 1996). This group includes sporadic disorders such as PSP, Pick's disease and corticobasal degeneration (CBD), and the familial autosomal dominant variants associated with FTDP-17 (Spillantini *et al.*, 1998). Other neurodegenerative disorders such as ALS/Parkinsonism-dementia complex (PDC) of Guam (ALS/PDC), AD, familial forms of prion disease, and familial British dementia also develop extensive Tau pathology and formation of NFTs with hyperphosphorylated Tau (Hutton *et al.*, 1998). Patients with FTDP-17 display mutations in the Tau protein accompanied by the formation of NFTs in the neocortex and limbic system, resulting in severe cognitive and motor alterations (Spillantini *et al.*, 1998). Based on these findings, tg models expressing wt and mutant variants of the Tau protein under the PrP and Thy-1 promoter have been generated (Hutton *et al.*, 2001).

4.2. Transgenic Models of Neurofibrillary Tangle Disease

Several tg models have been developed that express wt human isoforms of Tau, two of which overexpress the longest isoform of wt Tau under the regulatory control of the mThy-1 promoter (Table 13.2-3). These animals display somatodendritic localization of Tau in neurons as well as motor deficits in several assessment tests (Spittaels *et al.*, 1999; Probst *et al.*, 2000). These characteristics were accompanied by prominent axonopathy with dilated axons containing NF, tubulin, mitochondria, and vesicles in the brains of these mice. Hyperphosphorylated Tau immunoreactivity was present in dystrophic neurites, along with astrocyte activation and astrogliosis in the cortex. Despite the pathological alterations in the brains of these animals, they did not develop filamentous Tau inclusions, and there was no significant neuronal loss.

Table 13.2-3. Transgenic models of neurofibrillary pathology

Promoter	Construct	Mutation(s)	Phenotype	Reference(s)
mThy-1	2N4R Tau	wt	pretangles, gliosis, motor deficits, no neurofibrillary pathology	(Spittaels <i>et al.</i> , 1999; Probst <i>et al.</i> , 2000)
mThy-1	2N4R Tau	P301L	“neurofibrillary-like structures,” gliosis, no motor phenotype	(Gotz <i>et al.</i> , 2001b)
mThy-1, PDGFβ	2N4R Tau	V337M	PHFs present in hippocampus, mild behavioral deficits	(Tanemura <i>et al.</i> , 2001, 2002)
murine PrP	0N3R Tau	wt	rare NFTs in aged mice, motor deficits, insoluble Tau	(Ishihara <i>et al.</i> , 1999; Ishihara <i>et al.</i> , 2001a)
murine PrP	0N4R Tau	P301L	NFTs present in neurons, accompanied by gliosis, rare NFTs in cortex/hippocampus and severe motor deficits	(Lewis <i>et al.</i> , 2000)
murine PrP	Tau40	G272V	Tau phosphorylation in oligodendrocytes and fibrillary inclusions in spinal cord, no noticeable neurological deficits	(Gotz <i>et al.</i> , 2001c)
3H3McoA Reductase	0N3R Tau	wt	phosphorylated Tau and some pretangle neuropathology present	(Brion <i>et al.</i> , 1999)
PAC	genomic Tau	wt	lacks overall Tau pathological or biochemical changes	(Duff <i>et al.</i> , 2000)
α-CAMKII-tTA	2N4R Tau	R406W	hyperphosphorylated Tau inclusions present in forebrain neurons and insoluble Tau accumulates in aged mice	(Tatebayashi <i>et al.</i> , 2002)
α-CAMKII-tTA	GSK-3B (Tau kinase)	wt	hyperphosphorylated Tau, PHF immunoreactivity present	(Spittaels <i>et al.</i> , 1999)
rat NSE	P25 (Tau kinase)	wt	hyperphosphorylated Tau and NF, lacks neurofibrillary pathology	(Ahlijanian <i>et al.</i> , 2000)
mThy-1	PP2A (Tau kinase)	DN	hyperphosphorylation of endogenous Tau, some tau aggregates present	(Kins <i>et al.</i> , 2001)

3H3McoA, 3-hydroxy-3-methylglutaryl coenzyme A; DN, dominant negative.

In contrast to these two models expressing long isoforms of Tau, other models have been generated that overexpress the shortest wt Tau isoform under the control of the murine PrP promoter (T44 tg mice) (Ishihara *et al.*, 1999, 2001a) and the 3-hydroxy-3-methylglutaryl coenzyme A reductase promoter (Brion *et al.*, 1999). Protein-resistant prion protein-regulated Tau overexpression results in the development of NF-containing axonal spheroids in the spinal cord from 1 month of age, rare NFTs in aged mice, inclusions of Tau filaments, and some motor deficits (Ishihara *et al.*, 1999). Furthermore, insoluble Tau accumulated in the brains and spinal cords of these animals. In the second tg model, phosphorylated Tau was present with some pretangle neuropathology (Brion *et al.*, 1999).

In further studies of the T44 tg animals, these mice have been shown to develop abundant intraneuronal inclusions in the spinal cord with the presence of spheroids (Ishihara *et al.*, 2001b). These animals display motor defects, and the inclusions in the motor neurons are positive for hyperphosphorylated Tau and NFs. Ultrastructural analysis revealed the presence of 10–20-nm straight filaments in myelinated spinal cord axons. Taken together, the neuropathological alterations in the

spinal cord accompanied by the accumulation of insoluble Tau and motor deficits suggest that this model is highly reminiscent of ALS/PDC (Ishihara *et al.*, 2001b).

In another tg model of tauopathy, a P1-derived artificial chromosome (PAC) transgene containing the entire human Tau genome produces all six human isoforms of Tau (Duff *et al.*, 2000). Interestingly, this technique favors production of exon 10 and 3R Tau in the mouse brain. Human Tau immunoreactivity was present in the neuronal processes and synaptic terminals; however, these mice lacked any evidence of abnormal Tau pathology or behavioral changes up to 6 months of age. It is possible that this lack of Tau pathology is linked to the high proportion of 3R Tau production, or alternatively the Tau promoter in this model may only have diffuse expression compared to the promoters used in Tau cDNA tg mice.

In addition to tg models overexpressing wt isoforms of Tau, several models that express mutant forms of this molecule have been developed, and pathogenesis in these animals more closely resembles the pathology observed in human tauopathies (Table 13.2-3). One such model overexpresses the shortest isoform of Tau with the P301L mutation under the regulation of the mouse PrP promoter (Lewis *et al.*, 2000). These animals develop motor and behavioral deficits and NFTs with phospho-Tau immunoreactivity without NFs, show evidence of hyperphosphorylated Tau in brain tissue extracts, and display extensive neuronal loss in the spinal cord. In a second tg model overexpressing the longest isoform of Tau with the P301L mutation under the control of the mThy-1 promoter (Gotz *et al.*, 2001b), mice develop pretangles and some neurofibrillary-like structures (**Figure 13.2-2H; see color insert**), accompanied by astrogliosis and neuronal apoptosis. Unlike the P301L mutation in the short isoform of Tau, these mice do not display any apparent motor deficits.

In another tg model overexpressing the longest isoform of Tau, the R406W mutation was utilized because this is the same mutation found clinically in FTDP-17 (Tatebayashi *et al.*, 2002). In this model, Tau is overexpressed under the control of the α -calcium-calmodulin-dependent kinase-II (α -CAMKII) promoter, and results in the development of hyperphosphorylated Tau inclusions in forebrain neurons. Insoluble Tau was present in the brains of aged mice, and these animals displayed associative memory impairment but no significant motor deficits. Similarly, an additional tg model has been developed that contains another mutation characteristic of FTDP-17 (V337M) (Tanemura *et al.*, 2001, 2002). The overexpression of the longest isoform of Tau with this mutation under the control of the PDGF β promoter results in the aggregation of Tau in these animals that closely resembles the pathogenic NFTs described in human neurodegenerative diseases. In these animals, Tau aggregates are present in the hippocampus and a reduction of hippocampal neural activity accompanied by behavioral abnormalities. Neurons containing these NFTs also display phosphorylated Tau and ubiquitin immunoreactivity, accumulation of RNA, and also exhibit morphological characteristics of degenerating neurons (Tanemura *et al.*, 2002).

With the success of the mutant Tau tg models at developing neurofibrillary pathology, in recent years, efforts have been focused at identifying kinases/phosphatases that modulate *in vivo* Tau hyperphosphorylation. In this regard, three different tg models have been developed, including mice overexpressing GSK-3 β under the control of the α -CAMKII-tTA promoter (Spittaels *et al.*, 1999), dominant negative PP2A under the control of the mThy-1 promoter (Kins *et al.*, 2001), and P25 under the regulation of the rat neuron specific enolase (NSE) promoter (Ahlijanian *et al.*, 2000) (Table 13.2-3). P25 is a calpain cleavage product of P35, the endogenous regulator of CDK5, a putative Tau kinase (Patrick *et al.*, 1999). P25 promotes hyperactivation of CDK5, and has been proposed to be abnormally increased in AD (Patrick *et al.*, 1999). Mice expressing these molecules display hyperphosphorylated Tau and formation of neurofilamentous inclusions. Some of these lesions are reminiscent of pretangles, and are reactive with the PHF1 antibody that identifies the phospho-epitope at Ser396/404. However, none of these mice develop overt NFTs, suggesting that further studies are necessary to develop animal models that more closely recapitulate

human tauopathies and elucidate the precise mechanisms involved in the pathogenesis of these disorders.

5. Lewy Body Dementia and PD

5.1. Role of α -Synuclein in the Pathogenesis of LBD

Lewy body disease is a heterogeneous group of disorders that includes PD and dementia with Lewy bodies (DLB) (Kosaka *et al.*, 1984; Hansen and Galasko, 1992; McKeith, 2000), and is characterized by degeneration of the dopaminergic system (Shastri, 2001), motor alterations (Braak *et al.*, 2002), cognitive impairment (Salmon *et al.*, 1989), and formation of LBs in cortical and sub-cortical regions (Trojanowski and Lee, 1998).

In recent years, new hope for understanding the pathogenesis of this disease has emerged. Specifically, several studies have shown that the synaptic protein α -synuclein (Iwai *et al.*, 1994) plays a central role in LBD pathogenesis. α -Synuclein is a 140-amino acid (aa) synaptic molecule originally identified in the human brain as the precursor protein of the non-A β component (NAC) of AD amyloid (Ueda *et al.*, 1993; Masliah *et al.*, 1996a; Iwai, 2000), and is known to belong to a family that includes β -synuclein (or phosphoneuroprotein 14) (Nakajo *et al.*, 1993; Jakes *et al.*, 1994), γ -synuclein (or breast carcinoma-specific factor) (Jia *et al.*, 1999), and synoretin (Surguchov *et al.*, 1999). The pivotal role of α -synuclein in LBD pathogenesis is supported by studies showing that: (1) this molecule accumulates in LBs (Spillantini *et al.*, 1997; Wakabayashi *et al.*, 1997; Takeda *et al.*, 1998), (2) mutations and duplication in the α -synuclein gene are associated with rare familial forms of parkinsonism (Polymeropoulos *et al.*, 1997; Kruger *et al.*, 1998; Singleton *et al.*, 2003), and (3) its expression in tg mice (Kahle *et al.*, 2000; Masliah *et al.*, 2000; van der Putten *et al.*, 2000; Lee *et al.*, 2002, 2004) and *Drosophila* (Feany and Bender, 2000) mimics several aspects of PD. Thus, the fact that accumulation of α -synuclein in the brain is associated with similar morphological and neurological alterations in species as diverse as humans, mice, and flies suggests that this molecule contributes to the development of LBD.

α -Synuclein is capable of self-aggregating to form both oligomers and fibrillar polymers with amyloid-like characteristics (Hashimoto *et al.*, 1998; Uversky and Fink, 2002; Uversky *et al.*, 2002) (Figure 13.2-1). Most recent evidence suggests that there might be both low molecular weight (MW) nontoxic oligomers that associate with the cell membrane as well as higher MW toxic oligomers (protofibrils) (Hashimoto *et al.*, 1998; Conway *et al.*, 2000; Rochet *et al.*, 2000). Association of nontoxic oligomers with components of the plasma membrane such as polyunsaturated fatty acids might play a role in synaptic plasticity (Perrin *et al.*, 2000). In contrast, higher MW toxic oligomers form protofibrils that can potentially damage the cell membrane (Narayanan and Scarlata, 2001; Volles *et al.*, 2001). The role of fibril formation in PD and related disorders is more controversial, but a number of studies suggest that fibrils might represent less toxic byproducts or even a cellular strategy to inactivate or isolate more toxic oligomers (Hashimoto *et al.*, 1998; Conway *et al.*, 2000; Rochet *et al.*, 2000). Oligomerization could occur in several stages including the formation of protofibrils, nucleation (Wood *et al.*, 1999), and fibril formation (Hashimoto *et al.*, 1998; Serpell *et al.*, 2000).

Because of the implications for better understanding the disease pathogenesis and development of new treatments, conditions promoting or blocking α -synuclein aggregation and toxic conversion are now being extensively studied in tg animals and *in vitro* models. In general terms, and for the purposes of this manuscript, the role of the following factors will be considered: (1) mutations associated with familial parkinsonism (Conway *et al.*, 1998; Narhi *et al.*, 1999); (2) posttranscrip-

tional modifications associated with oxidative stress mediated by neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), paraquat, iron, cytochrome *c*, copper (II), and dopamine (Hashimoto *et al.*, 1998, 1999b, 1999a; Paik *et al.*, 1999; Hsu *et al.*, 2000; Souza *et al.*, 2000); (3) posttranscriptional modifications associated with signaling events or conjugation (phosphorylation, glycosylation, ubiquitination) (Okochi *et al.*, 2000; Fujiwara *et al.*, 2002); (4) binding to lipid membrane vesicles (Davidson *et al.*, 1998; Jo *et al.*, 2000; Perrin *et al.*, 2000); and (5) interactions with molecules that promote aggregation (e.g., α -synuclein and A β) (Yoshimoto *et al.*, 1995; Jensen *et al.*, 1997; Paik *et al.*, 1998; Masliah, 2000) or that block aggregation (e.g., β -synuclein) (Hashimoto *et al.*, 2001).

5.2. α -Synuclein tg Models of LBD

Because progressive intraneuronal aggregation of α -synuclein has been proposed to play a central role in the pathogenesis of PD and related disorders (Hashimoto and Masliah, 1999; Trojanowski and Lee, 2000; Volles and Lansbury, 2002), most tg models have been focused on investigating the *in vivo* effects of α -synuclein accumulation utilizing neuron-specific promoters (Table 13.2-4). Several recent reviews have been published recently dealing with this subject (Hashimoto *et al.*, 2003; Fernagut and Chesselet, 2004). Among these models, overexpression of

Table 13.2-4. Models of Lewy body disease

Promoter	Construct	Mutation(s)	Phenotype	Reference(s)
PDGF β	h α -synuclein	wt	α -synuclein inclusions, DOPA loss, mild motor deficits, increased α -synuclein aggregates	(Masliah <i>et al.</i> , 2000)
PDGF β	h α -synuclein	A53T	α -synuclein accumulation in synapses and neurons, few inclusions but progressive motor deficits and neurodegeneration	(Hashimoto <i>et al.</i> , 2003)
murine PrP	h α -synuclein	wt, A53T, A30P	severe motor deficits, detergent insoluble α -synuclein, axonal degeneration, premature death, gliosis	(Lee <i>et al.</i> , 2002)
murine PrP	h α -synuclein	wt, A53T, homozygous	α -synuclein accumulation, severe motor deficits, detergent insoluble α -synuclein, axonal degeneration, gliosis	(Giasson <i>et al.</i> , 2002)
mThy-1	h α -synuclein	wt, A53T	early onset motor deficits, axonal degeneration	(van der Putten <i>et al.</i> , 2000)
mThy-1	h α -synuclein	wt, A30P	accumulation of α -synuclein and increased aggregates	(Rockenstein <i>et al.</i> , 2002)
mThy-1	h α -synuclein	A30P	mutant h α -synuclein in synapses, Lewy body characteristics present	(Kahle <i>et al.</i> , 2000)
PLP	h α -synuclein	wt	accumulation of α -synuclein oligonucleotides and detergent insoluble aggregates	(Kahle <i>et al.</i> , 2002)
rat TH	h α -synuclein	A30P	accumulation of α -synuclein in TH-positive neurons in SN	(Rathke-Hartlieb <i>et al.</i> , 2001)
rat TH	h α -synuclein	wt, A53T, A30P	accumulation of α -synuclein in SN neurons, no neurodegeneration or motor deficits	(Richfield <i>et al.</i> , 2002)
rat TH	h α -synuclein	wt, A53T, A30P	α -synuclein in SN	(Matsuoka <i>et al.</i> , 2001)

PLP, proteolipid protein promoter.

wt α -synuclein under the regulatory control of the PDGF β promoter has been shown to result in motor deficits, dopaminergic loss, and the formation of inclusion bodies (Masliah *et al.*, 2000). Mice with the highest levels of expression (Line D) showed intraneuronal accumulation of α -synuclein that started at 3 months of age (**Figure 13.2-2I**; see color insert), and was accompanied by loss of tyrosine hydroxylase (TH) fibers in the caudoputamen region and synapses in the temporal cortex. Although no apparent neuronal loss was detected in the substantia nigra (SN), measurements of dopamine levels in the caudoputamen region showed a 25–50% decrease at 12 months of age. Analysis of locomotor activity in the open field showed that these tg mice displayed increased thigmotaxis—a behavior that has been associated with dopaminergic activity. Supporting a role for alterations in the dopaminergic system, this abnormal behavior was ameliorated upon treatment with apomorphine—a compound known to promote dopamine release. Furthermore, tg mice showed mild to moderate motor deficits in the rotarod, particularly in mice with the greatest loss of dopamine, indicating that more substantial loss (>75%) of this transmitter might be necessary for more overt deficits to appear.

To determine whether different levels of h α -synuclein expression from distinct promoters might result in neuropathology mimicking other synucleopathies, we also compared patterns of h α -synuclein accumulation in the brains of tg mice expressing this molecule from the mThy-1 and PDGF β promoters (Rockenstein *et al.*, 2002). In mThy-1-h α -synuclein tg mice, this protein accumulated in synapses and neurons throughout the brain, including the thalamus, basal ganglia, SN, and brainstem. Expression of h α -synuclein from the PDGF β promoter resulted in accumulation in synapses of the neocortex, limbic system, and olfactory regions as well as formation of inclusion bodies in neurons in deeper layers of the neocortex (Lines A and D). Furthermore, one of the intermediate expresser lines (Line M) displayed h α -synuclein expression in glial cells, mimicking some features of multiple system atrophy (MSA). These results show a more widespread accumulation of h α -synuclein in tg mouse brains. Taken together, these studies support the contention that h α -synuclein expression in tg mice might mimic some neuropathological alterations observed in LBD and other synucleopathies, such as MSA (Rockenstein *et al.*, 2002).

However, from these studies, it was still unclear what was the potential relationship among synaptic damage, α -synuclein oligomerization and fibrillation, inclusion formation, and neurological deficits. Because previous studies have shown that mutations associated with familial parkinsonism accelerate α -synuclein aggregation and oligomerization (Conway *et al.*, 1998; Narhi *et al.*, 1999), we compared the patterns of neurodegeneration, α -synuclein aggregation and neurological alterations in tg mice expressing wt or mutant (A53T) h α -synuclein at comparable levels under the PDGF β promoter. For this purpose two tg lines that expressed moderate levels of α -synuclein in a similar anatomical and cellular distribution were compared: Line A mice that expressed wt h α -synuclein (Masliah *et al.*, 2000; Rockenstein *et al.*, 2002) and a newly developed Line 8 that expressed mutant h α -synuclein (A53T) (Hashimoto *et al.*, 2003). Additional comparisons were done against our well-described Line D mice that express higher levels of wt h α -synuclein (Masliah *et al.*, 2000; Rockenstein *et al.*, 2002). Lower expresser lines were selected for these experiments to avoid unintended effects of very high levels of expression as observed with other stronger promoters such as Thy-1 and PrP. These mice express about a third of the levels of h α -synuclein compared to our higher expresser Line D, which is approximately equivalent to a 1.5:1 ratio with respect to endogenous levels. Remarkably, we found that mice expressing mutant h α -synuclein developed progressive motor deficits and neurodegeneration associated with h α -synuclein accumulation in synapses and neurons, but very few or no inclusions were found. In contrast, mice from Line A (wt h α -synuclein) did not show neurodegenerative or neurological deficits, but display formation of inclusions. Analysis of the patterns of α -synuclein aggregation by Western blot using the Syn-1 antibody showed that in the mutant line there was greater formation of α -synuclein oligomers compared to the wt line. This is

consistent with recent studies comparing the neuropathogenic effects of human wt and mutant α -synuclein under the control of the PrP promoters (Table 13.2-4). For example, mice expressing mutant, but not wt, α -synuclein developed a severe and complex motor impairment leading to paralysis and death (Giasson *et al.*, 2002). These animals developed age-dependent intracytoplasmic neuronal α -synuclein inclusions paralleling disease onset, and the α -synuclein inclusions recapitulated features of human disorders. Moreover, immunoelectron microscopy revealed that the α -synuclein inclusions contained 10–16 nm wide fibrils similar to human pathological inclusions. These mice demonstrate that A53T α -synuclein leads to the formation of toxic filamentous α -synuclein neuronal inclusions that cause neurodegeneration (Giasson *et al.*, 2002).

Similarly, other studies have shown that under the PrP promoter, mice expressing the A53T h α -synuclein, but not wt or the A30P variants, develop adult-onset neurodegenerative disease with a progressive locomotor dysfunction leading to death (Lee *et al.*, 2002) (Table 13.2-4). Affected mice exhibit neuronal abnormalities (in perikarya and neurites) including pathological accumulations of α -synuclein and ubiquitin. Consistent with abnormal neuronal accumulation of α -synuclein, brain regions with pathology exhibit increases in detergent-insoluble α -synuclein and α -synuclein aggregates (Lee *et al.*, 2002).

Under the mThy-1 promoter, expression of either wt or mutant α -synuclein (van der Putten *et al.*, 2000) results in extensive α -synuclein accumulation throughout the CNS, including in some cases in the SN or the motor neurons (Rockenstein *et al.*, 2002) (Table 13.2-4). In the model developed by van der Putten *et al.*, mice develop early onset motor decline, axonal degeneration, and α -synuclein accumulation in the spinal cord but not in the SN. In contrast, in our models we observed less overt motor deficits, but considerable accumulation of α -synuclein in cortical and subcortical regions (Rockenstein *et al.*, 2002). In both models there is accumulation of detergent insoluble α -synuclein; however, its effects in the nigral system in terms of cell death are not completely clear.

To address the question of selective neuronal vulnerability in tg mice expressing either wt or mutated (A53T or A30P) forms of h α -synuclein under control of the 9-kb rat TH promoter have been developed (Richfield *et al.*, 2002) (Table 13.2-4). Initial studies in these mice showed accumulation of α -synuclein in the SN neurons but no neurodegeneration or motor deficits. More recent studies in tg mice expressing either wt or a doubly mutated forms of h α -synuclein under control of the rat TH promoter have shown that the expression of h α -synuclein in nigrostriatal terminals resulted in increased density of the dopamine transporter and enhanced toxicity to the neurotoxin MPTP. Expression of a double mutant h α -synuclein reduced locomotor responses to repeated doses of amphetamine and blocked the development of sensitization compared to adult wt h α -synuclein tg mice. Taken together, these results indicate that expression of double mutant h α -synuclein adversely affects the integrity of dopaminergic terminals and leads to age-related declines in motor coordination and dopaminergic markers (Richfield *et al.*, 2002).

Further studies to investigate the role of α -synuclein mutations and selective neuronal vulnerability in the SN has been performed in rats utilizing lentiviral and adeno-associated viral vectors (Kirik *et al.*, 2002; Klein *et al.*, 2002; Lo Bianco *et al.*, 2002) (Table 13.2-4). In contrast to tg mice models, a selective loss of nigral dopaminergic neurons associated with a dopaminergic denervation of the striatum was observed in animals expressing either wt or mutant forms of h α -synuclein. This neuronal degeneration correlates with the appearance of abundant α -synuclein-positive inclusions and extensive neuritic pathology detected with both α -synuclein and silver staining. Rat α -synuclein similarly leads to protein aggregation but without cell loss, suggesting that inclusions are not the primary cause of cell degeneration in PD (Lo Bianco *et al.*, 2002).

In summary, under the PDGF β promoter both wt and mutant α -synuclein tg mice develop behavioral deficits and synaptic alterations following a limbic and cortical pattern; however, mutant α -synuclein is more toxic despite the fact that very few inclusions are formed compared to wt α -

synuclein tg mice. Under the control of the PrP promoter, mutant α -synuclein is more toxic than wt, inclusion-like structures develop, and more widespread involvement of the lower motor system is observed. With the Thy-1 construct, both wt and mutant α -synuclein are toxic, and inclusion-like structures develop that can affect cortical or subcortical regions or the spinal cord. Furthermore, these *in vivo* models support the contention that α -synuclein-dependent neurodegeneration is associated with abnormal accumulation of detergent-insoluble α -synuclein (probably representing oligomeric forms) rather than with inclusion formation representing fibrillar polymeric α -synuclein. The specific accumulation of detergent-insoluble α -synuclein in these tg mice recapitulates a pivotal feature of LBD (Kahle *et al.*, 2001), and it is of significant importance in the future development and evaluation of novel treatments.

5.3. Modeling Other Environmental and Genetic Factors in LBD tg Models

Although α -synuclein is centrally involved in the pathogenesis of PD, and characterizing the role of α -synuclein mutations has led to better understanding of the disease pathogenesis, these mutations compose only a small fraction of the cases. In fact, in familial forms of PD and LBD, α -synuclein mutations are very rare, and mutations within several other genes have been recently reported. Among them, the most common are the Parkin mutations followed by PINK-1, DJ-1, UCH-L1, Nurr1, and β -synuclein (Vila *et al.*, 2000; Bossy-Wetzel *et al.*, 2004; McInerney-Leo *et al.*, 2005; Valente *et al.*, 2004). Although Parkin and UCH-L1 play a role in ubiquitination and proteosomal degradation, PINK-1 and DJ-1 are involved in mitochondrial function among other potential roles (Vila *et al.*, 2000; Dawson and Dawson, 2003; Bossy-Wetzel *et al.*, 2004; Valente *et al.*, 2004). Nurr1 has been shown to be an important transcriptional factor for dopaminergic cell differentiation (Wallen and Perlmann, 2003) and β -synuclein is a regulator of synaptic plasticity with the capacity of preventing α -synuclein aggregation (Hashimoto *et al.*, 2001). Most of the mutations within the Parkin, UCH-L1, DJ-1, and PINK-1 genes lead to loss of function, although gain of some toxic activity might also be at play. For this reason, studies are currently underway involving the crossing of α -synuclein tg with Parkin, UCHL-1, and DJ-1-deficient mice. The prediction is that this will result in defective α -synuclein ubiquitination and proteosome degradation with worsening of the phenotype, but probably with no inclusion formation. However, it is possible that deficient expression of these molecules might not be sufficient, and that overexpression of the mutant Parkin, UCHL-1, DJ-1, or PINK-1 genes in the KO background might be necessary to develop more comprehensive models of PD and LBD.

It is estimated that over 95% of the cases of PD and related disorders are sporadic (Goedert, 2001). Thus, complex interactions between environmental and genetic susceptibility factors might be at play. Among them, the potential role of factors promoting oxidative stress is under intense investigation. Factors such as iron, cytochrome *c*, copper (II), dopamine (Hashimoto *et al.*, 1998, 1999a, 1999b; Paik *et al.*, 1999; Giasson *et al.*, 2000; Hsu *et al.*, 2000; Souza *et al.*, 2000; Conway *et al.*, 2001) and neurotoxins have been shown *in vitro* to alter α -synuclein conformation, leading to aggregation and toxic protofibril formation. Among such factors, the key discovery by Langston and colleagues of 1-methyl-4-phenylpyridinium ion (MPP⁺) as a powerful dopaminergic neurotoxin led to the development of acute models of parkinsonism in murine and simian models (Langston *et al.*, 1984).

Because the neurotoxin MPTP promotes oxidative stress (Grunblatt *et al.*, 2000) and degeneration of the dopaminergic system mimics some aspects of PD, several groups have investigated the effects of chronic MPTP treatment on tg mice overexpressing α -synuclein or in α -synuclein KO mice. Interestingly, deletion of the α -synuclein gene prevented the neurotoxic (Drolet *et al.*, 2004) effects

of MPTP, in contrast to α -synuclein overexpression, which exacerbates MPTP mitochondrial injury. Transgenic mice expressing high levels of α -synuclein throughout the cortex and SN under the control of the Thy-1 promoter were treated with low doses of MPTP and analyzed ultrastructurally (Rathke-Hartlieb *et al.*, 2001; Song *et al.*, 2004). Electron microscopy analysis demonstrated that compared to control groups, Thy1- α -synuclein tg mice treated with MPTP showed extensive mitochondrial alterations, invagination of the nuclear membrane, and formation of electron-dense intracytoplasmic inclusions. Taken together, these studies suggest that oxidative stress and associated mitochondrial damage might contribute to aggregation of α -synuclein and to the pathogenesis of PD. Further supporting a role for oxidative stress in the pathogenesis of disorders with α -synuclein accumulation, recent studies have shown that this molecule is nitrosylated, promoting abnormal conformation (Giasson *et al.*, 2000; Souza *et al.*, 2000). Consistent with this possibility, we have observed increased 3-nitrotyrosine (3NT) immunostaining in α -synuclein tg mice. In neuronal cell lines overexpressing α -synuclein, there is also increased reactivity for markers of oxidative stress that are reversible with antioxidant treatments such as Vitamin E (Hsu *et al.*, 2000). More recently, significant interest has developed in investigating the interactions between α -synuclein and other pesticides and neurotoxins such as rotenone and paraquat (Di Monte, 2003; Greenamyre *et al.*, 2003).

Other factors promoting oxidative damage and abnormal protein conformation that might play a role in synucleopathies include A β protein. This is interesting because AD and PD are associated with the cerebral accumulation of A β and α -synuclein, respectively (Masliah *et al.*, 2001c). Some patients have clinical and pathological features of both diseases, raising the possibility of overlapping pathogenic pathways. We refer to this condition as the Lewy body variant of AD (LBV) (Hansen *et al.*, 1990); however, others prefer different terms such as combined AD + PD, senile dementia of the Lewy body type or LBD (McKeith *et al.*, 1996). To better understand the role of amyloid production in synucleopathies, we generated tg mice with neuronal expression of human A β , α -synuclein, or both (Masliah *et al.*, 2001c). The functional and morphological alterations in doubly-tg mice resembled the LBV of AD. These mice had severe deficits in learning and memory, developed motor deficits earlier than α -synuclein singly-tg mice, and showed prominent age-dependent degeneration of cholinergic neurons and presynaptic terminals. They also had more α -synuclein-immunoreactive neuronal inclusions than α -synuclein singly-tg mice. Ultrastructurally, some of these inclusions were fibrillar in doubly-tg mice, whereas all inclusions were amorphous in α -synuclein singly-tg mice. A β promoted aggregation of α -synuclein in a cell-free system and also promoted intraneuronal accumulation of α -synuclein in cell culture. A β may contribute to the development of LBD by promoting the aggregation of α -synuclein and exacerbating α -synuclein-dependent neuronal pathologies. Therefore, treatments that block the production or accumulation of A β could benefit from a broader spectrum of disorders than previously anticipated.

Other posttranscriptional modifications that might promote α -synuclein aggregation and toxic conversion include phosphorylation and conjugation. In this regard, recent mass spectrometry analysis and studies with an antibody that specifically recognizes the phospho-Ser129 form of α -synuclein have shown that this residue is selectively and extensively phosphorylated in synucleinopathy lesions including patients with PD and related disorders and α -synuclein tg mice (Fujiwara *et al.*, 2002). Furthermore, phosphorylation of α -synuclein at Ser129 promoted fibril formation *in vitro*. These results highlight the importance of phosphorylation of filamentous proteins in the pathogenesis of neurodegenerative disorders. For this reason new models of PD and LBD will include overexpression of mutant forms of synuclein that might facilitate Ser129 phosphorylation or other posttranscriptional changes such as cleavage (C110 fragment) or oxidation (Lee *et al.*, 2004). In *Drosophila*, synuclein overexpression results in LB formation and dopaminergic neuronal loss (Feany and Bender, 2000). Furthermore, directed expression of the molecular chaperone Hsp70 prevented dopaminergic neuronal loss associated with α -synuclein in *Drosophila* and interference with endogenous chaperone

activity accelerated α -synuclein toxicity (Auluck *et al.*, 2002). Similarly, in mammalian models, introducing the molecular chaperone Hsp70 into the *in vivo* model by breeding α -synuclein tg mice with Hsp70 overexpressing mice led to a significant reduction in both the high molecular weight and detergent insoluble α -synuclein species (Klucken *et al.*, 2004). Furthermore, Hsp70 overexpression *in vitro* reduced detergent insoluble α -synuclein species and protected cells from α -synuclein-induced cellular toxicity (Klucken *et al.*, 2004). Taken together, these data demonstrate that the molecular chaperone Hsp70 can reduce the amount of misfolded, aggregated α -synuclein species *in vivo* and *in vitro*, and protect from α -synuclein-dependent toxicity. Because LBs in human postmortem tissue immunostained for molecular chaperones, this suggests that chaperones may play a role in PD progression. Among them, ubiquitination appears to play an important role. This is significant because mutations in the Parkin gene, also known to be associated with familial PD, have been shown to result in deficient α -synuclein ubiquitination (Hattori *et al.*, 1998; Kitada *et al.*, 1998; Shimura *et al.*, 2000).

More recently it has been identified that β -synuclein is also a molecular chaperone of α -synuclein, and might also regulate the state of aggregation and toxicity of this molecule (Hashimoto *et al.*, 2001) and regulate synaptic transmission. Overexpression of β -synuclein in α -synuclein tg mice ameliorated motor deficits, neurodegenerative alterations, and neuronal α -synuclein accumulation (Hashimoto *et al.*, 2001). Similarly, cell lines transfected with β -synuclein were resistant to α -synuclein accumulation. α -Synuclein was coimmunoprecipitated with β -synuclein in the brains of doubly-tg mice and in the double-transfected cell lines (Hashimoto *et al.*, 2001). Furthermore, delivery of lentiviral vectors overexpressing β -synuclein in tg mice reduced the α -synuclein related toxicity by regulating Akt activity, a signaling pathway involved in neuronal survival (Hashimoto *et al.*, 2004). Thus, β -synuclein might be a natural negative regulator of α -synuclein aggregation and a similar class of endogenous factors might regulate the aggregation state of other molecules involved in neurodegeneration. Such an anti-amyloidogenic property of β -synuclein might also provide a novel strategy for the treatment of neurodegenerative disorders.

6. Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis is the most common form of adult onset motor neuron disease. It is characterized by selective loss of upper and lower motor neurons in the cerebral cortex and spinal cord resulting in spasticity, muscle weakness, and atrophy. Approximately 10% of ALS cases are familial and 90% are sporadic (Jackson and Bryan, 1998). Between 15 and 20% of the familial cases are due to mutations in the SOD1 gene (Deng *et al.*, 1993; Rosen *et al.*, 1993) that are generally inherited in an autosomal dominant manner (Orrell *et al.*, 1996). Several tg mouse models that express different mutated variants of the SOD1 gene under the regulatory control of the SOD1 and other promoters have been generated (Gurney *et al.*, 1994; Dal Canto and Gurney, 1995; Ripps *et al.*, 1995; Wong *et al.*, 1995; Bruijn *et al.*, 1997; Friedlander *et al.*, 1997) (detailed in Shibata, 20001). These mice develop early severe motor dysfunction with paralysis of the lower limbs accompanied by SOD1 and ubiquitin positive aggregates in astrocytes and neurons (**Figure 13.2-2K; see color insert**) in the spinal cord. Because crossbreeding these mice into an endogenous mSOD1-deficient background did not affect the progression of the clinical and neuropathological features, it is possible that neurodegeneration is associated with gain of toxicity rather than loss of antioxidant function of the mutant SOD1 protein (Bruijn *et al.*, 1998). Similar to other murine models of neurodegeneration, in the mutant SOD1 tg mice there is accumulation of misfolded oligomers of SOD1 prior to the formation of inclusions containing polymeric (fibrillar) forms of the pathogenic molecule (Wood *et al.*, 2003).

In addition to tg mice expressing mutant forms of the SOD1 gene, overexpression of molecules involved in cytoskeletal development and function in combination with SOD1 mutations provide

interesting animal models of ALS pathogenesis. Included are mice overexpressing various subunits of NF proteins (Cote *et al.*, 1993; Xu *et al.*, 1993; Meier *et al.*, 1999) and peripherin (Beaulieu *et al.*, 1999, 2000), as well as animals containing deletions in the vascular endothelial growth factor (VEGF) promoter (Oosthuysen *et al.*, 2001) and in the Eukaryotic elongation factor 1A-2 (Eef1a2) in the wasted (wst) model (Chambers *et al.*, 1998). In animals that overexpress NFs at various levels, it is unclear whether NF subunit overexpression is actually detrimental or protective against development of ALS characteristics; however, correlations have been made between the severity of symptoms and NF-H levels and overexpression of NF-L and a rescuing of the diseased phenotype in animals (Cote *et al.*, 1993; Meier *et al.*, 1999). Taken together, these models provide insight into the pathogenesis and potential therapeutic approaches for ALS.

7. Neurodegenerative Disorders with Trinucleotide Repeats

7.1. Introduction to the Pathogenesis of Trinucleotide Repeat Disorders in Animal Models

Neurodegenerative disorders with TNRs include hereditary neurological conditions where the repeats are either coded into polyglutamine or represent uncoded triplet repeat expansions. Nine neurodegenerative disorders are known to be caused by expanding CAG repeats coding for polyglutamine (Margolis and Ross, 2001), including Spinal and Bulbar Muscular Atrophy (SBMA), HD, DentatoRubral. and PallidoLusian Atrophy, and several forms of Spino-Cerebellar Ataxias (SCA). Each of these disorders is characterized by localized neuronal cell death in specific brain regions, including the basal ganglia, brainstem nuclei, cerebellum, and spinal motor nuclei (Ross, 1995). In all polyglutamine repeat diseases, there is a common threshold effect of the minimum polyglutamine length necessary to cause disease. Although the pathogenesis of coding TNRDs usually is characterized by autosomal dominant inheritance and the gain of a protein function, noncoding triplet repeat disorders, including fragile-X mental retardation and Friedreich's ataxia, are recessive and consistent with a loss of function mechanism.

7.2. Models of HD

Of the coding TNRDs, HD is one of the best characterized. Several HD mouse models have been published to date (reviewed in Menalled and Chesselet, 2002) (Table 13.2-5); the first described and most studied of these is the R6/2 tg mouse generated using the human HD IT15 promoter with a construct overexpressing exon-1 of the HD gene containing a 150 CAG repeat expansion (Mangiarini *et al.*, 1996; Carter *et al.*, 1999). These mice develop a degenerative neurological disorder associated with progressive weight loss and recapitulate many of the features of the human disease (**Figure 13.2-2L; see color insert**). These were also the first HD model in which nuclear inclusions of huntingtin were detected in neurons as early as 7 weeks of age. In addition to several models expressing fragments of the HD gene, others expressing the full-length human huntingtin gene have been developed, including the HD16, HD48, and HD89 animals (Reddy *et al.*, 1998, 1999) (Table 13.2-5). Mice expressing a lentivirally delivered mutated form of human huntingtin under control of the Cytomegalovirus (CMV) promoter show a progressive motor phenotype and neuronal loss in the striatum (de Almeida *et al.*, 2002), providing a model for selective neural degeneration that may support future studies on the pathogenesis of cell death and experimental therapeutics for HD. This collection of animal models provides insight into the detrimental function of expanded genes encoding for polyglutamine.

Table 13.2-5. Transgenic models of TNRDs

Promoter	Construct	Number of TNRs	Phenotype	Reference(s)
IT15	exon 1 of huntingtin (R6)	142, 115, 145, 128–156	HD model: early motor deficits, reduced brain weight and progressive overall weight loss, lack of apoptosis	(Mangiarini <i>et al.</i> , 1996; Carter <i>et al.</i> , 1999)
CMV	full-length huntingtin	48, 89	HD model: progressive motor deficits and neuronal loss in the striatum	(Reddy <i>et al.</i> , 1998)
YAC	full-length huntingtin	72	HD model: cell loss limited to striatum, no nuclear inclusions	(Hodgson <i>et al.</i> , 1999)
NSE	tissue-specific frataxin deficiency	NA	Freidreich's ataxia model: low birth weight, progressive neurological phenotype and ataxia, premature death, and low death weight	(Puccio <i>et al.</i> , 2001)
PCP2/L7	ataxin1	82	SCA1 model: progressive Purkinje cell pathology accompanied by severe ataxia;	(Burright <i>et al.</i> , 1995; Clark and Orr, 2000)
L7	ataxin3 fragment	79	SCA3 model: early gait disturbance, ataxic posture and atrophy of cerebellum	(Ikeda <i>et al.</i> , 1996)
YAC	ataxin3	76, 84	SCA3 model: mild and slowly progressive cerebellar deficits	(Cemal <i>et al.</i> , 1999, 2002)
PCP2	full-length ataxin7	90	SCA7 model: formation of ubiquitinated nuclear inclusions accompanied by a late onset motor deficiency	(Yvert <i>et al.</i> , 2000)

PCP2, Purkinje cell-specific promoter.

7.3. Models of Noncoding Trinucleotide Repeat Disorders

Noncoding triplet repeat expansions, including Fragile X syndrome and Friedreich's ataxia, were among the first TNRDs described (Table 13.2-5). Friedreich's ataxia is the most common autosomal recessive ataxia in Caucasians, and is typically caused by a noncoding GAA repeat expansion in the first intron of the gene encoding the mitochondrial protein frataxin. Homozygous frataxin KO mice die early in embryogenesis (Cossee *et al.*, 2000), demonstrating an important role for frataxin in mouse development. Through a conditional gene-targeting approach, Puccio *et al.* (2001) generated a neuronal frataxin-deficient line, which reproduced some of the progressive pathophysiological and biochemical features of human ataxia. Recently, a new model expressing 25–36% of wt frataxin has been reported; these mice are phenotypically normal, showing that low levels of the normal protein may be compensatory (Miranda *et al.*, 2002).

In addition to Friedreich's ataxia, several variants of autosomal dominantly inherited SCA have been identified. Types 1, 2, and 3 fit the classical concept of neurological disease caused by a poly-glutamine expansion (Orr and Zoghbi, 2001), whereas types 8, 10, and 12 correspond to the group of neurodegenerative diseases in which mutations in microsatellite expansions exert their pathogenic effect at the RNA level (Ranum and Day, 2004). The causative genetic mutation has been identified in SCA1, 2, 3, 6, 7, 8, 10, 12, and 17 (reviewed in Wullner, 2003). Transgenic models of SCA1 (Burright *et al.*, 1995; Clark and Orr, 2000), SCA3 (also known as Machado-Joseph disease, or MJD) (Ikeda *et al.*, 1996; Cemal *et al.*, 1999, 2002), and SCA7 (Yvert *et al.*, 2000) have been reported, and all these models exhibit a progressive gait ataxia with cerebellar degeneration, thus modeling

many features of the clinical phenotype seen in patients. Taken together, these models of human SCA provide insight into the pathogenesis of both coding and noncoding TNRDs.

8. Conclusions

Several tg mouse models of neurodegenerative disorders such as AD, LBD/PD, FTD, TNRDs, and others have now been developed. Most of these models reproduce one or several clinical, pathological and molecular aspects of these diseases. The levels of transgene expression, the type of promoter, mutations, and genetic background of the mice are important determinants for the pathological phenotype. Models employing the PDGF β promoter display involvement of the neocortex, limbic system, and to a lesser extent, the subcortical structures. In contrast, under the mThy-1 and PrP promoters there is more widespread expression of the transgene throughout the brain including the sub-cortex and spinal cord. In these animals there is a more dramatic clinical and pathological phenotype and increased lethality with involvement of the neuromuscular junction and motor system. Other neuron specific promoters (e.g., TH, NF, and NSE) have proven to be less effective, probably because of low levels of expression. Several of these transgenes, as well as cytokines and chemokines have also been expressed in the CNS under the regulatory control of glial cell promoters (e.g., GFAP), leading to the development of novel models of neurodegenerative and neuroinflammatory disorders (reviewed in Campbell, 1998; Campbell *et al.*, 1998).

Other promoters under testing include genomic constructs using yeast vectors. In addition to the more traditional murine models, new attempts have been focused at developing knock-in and combined tg and KO models, or at expressing certain transgenes under the control of inducible promoters. Some progress has been made in this respect, particularly in the development and testing of inducible models for prion diseases (Tremblay *et al.*, 1998) and HD (Menalled and Chesselet, 2002). The inducible HD model (HD94) utilized a tetracycline-inducible system (tet-off) to control the expression of a chimeric mouse/human exon 1 containing 94 repeats of CAG (Yamamoto *et al.*, 2000). When expression of the mutant huntingtin was induced at birth, the mice showed progressive motor dysfunction, as well as an HD-like neuropathology. For the other neurodegenerative disorders these models have been proven more difficult to develop.

A novel approach toward the development of models of neurodegeneration in mammals includes the use lentiviral and adeno-associated viral vectors. These vectors can be injected into the eggs or directly into the brains of mature animals including mice, rats, and macaques with excellent results (Verma and Gage, 2000; Lo Bianco *et al.*, 2002). Crosses of tg models of neurodegeneration with other models expressing antioxidant, pro-inflammatory, antiapoptotic or molecular chaperones have been developed to test new possible treatments and mechanisms of disease. Overall, most of these studies suggest that oligomers and protofibrils rather than fibrillar structures and inclusions are responsible for the pathogenesis of the neurodegenerative process. Thus, it is important when developing these models not only to demonstrate a wide range of clinical and neuropathological features but also the formation of oligomers, which in the end are the primary target for therapy. These models hold the promise for development and discovery of new treatments for these diseases.

9. Abbreviations

aa	amino acid
A β	amyloid- β peptide
AD	Alzheimer's disease

α -CAMKII	α -calcium-calmodulin-dependent kinase-II
ALS	Amyotrophic Lateral Sclerosis
ALS/PDC	ALS/Parkinsonism-dementia complex (PDC) of Guam
apoE	apolipoprotein E
APP	amyloid precursor protein
CAA	cerebral amyloid angiopathy
CBD	corticobasal degeneration
CJD	Creutzfeld-Jacobs disease
CNS	central nervous system
DLB	dementia with Lewy bodies
Eef1a2	Eukaryotic elongation factor 1A-2
ER	endoplasmic reticulum
FAD	familial AD
FGF2	fibroblast growth factor 2
FTD	Fronto-Temporal Dementias
FTDP-17	FTD with parkinsonism linked to chromosome 17
h	human
hACT	α 1-antichymotrypsin
HD	Huntington's disease
HCHWA	hereditary cerebral hemorrhage with amyloidosis
KO	knockout
LBs	Lewy bodies
LBD	Lewy body disease
LBV	Lewy body variant of AD
LTP	long-term potentiation
LRP	low-density lipoprotein receptor-related protein
m	murine
MPP +	1-methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger RNA
MSA	multiple system atrophy
MW	molecular weight
3NT	3-nitrotyrosine
NAC	non-A β component
NFs	neurofilaments
NF-L	low molecular weight NF
NFTs	neurofibrillary tangles
nontg	nontransgenic
NSE	neuron specific enolase
PD	Parkinson's disease
PDAPP	PDGF β promoter drives an alternatively spliced hAPP minigene
PDGF β	platelet-derived growth factor (β chain)
PHFs	paired-helical filaments
PrP	protease-resistant prion protein
PS	presenilin
PSP	progressive supranuclear palsy
RAP	receptor-associated protein
SBMA	Spinal and Bulbar Muscular Atrophy

SCA	Spino-Cerebellar Ataxias
sCrry	soluble complement receptor-related protein γ
SN	substantia nigra
tg	transgenic
TGF β 1	transforming growth factor β 1
TH	tyrosine hydroxylase
TNRD	trinucleotide repeat disorders
VEGF	vascular endothelial growth factor
wst	wasted
wt	wild type

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References

- Abraham, C.R., McGraw, W.T., Slot, F., and Yamin, R. (2000). Alpha 1-antichymotrypsin inhibits A beta degradation in vitro and in vivo. *Ann. N. Y. Acad. Sci.* 920:245–248.
- Aguzzi, A., and Raeberm A.J. (1998). Transgenic models of neurodegeneration. Neurodegeneration of (transgenic) mice and men. *Brain Pathol.* 8:695–697.
- Aguzzi, A., Brandner, S., Marino, S., and Steinbach, J. (1996). Transgenic and knockout mice in the study of neurodegenerative diseases. *J. Mol. Med.* 74:111–126.
- Ahljani, M.K., Barrezaeta, N.X., Williams, R.D., Jakowski, A., Kowsz, K.P., McCarthy, S., Coskran, T., Carlo, A., Seymour, P.A., Burkhardt, J.E., Nelson, R.B., and McNeish, J.D. (2000). Hyperphosphorylated tau and neurofilament and cytoskeletal disruptions in mice overexpressing human p25, an activator of cdk5. *Proc. Natl. Acad. Sci. USA* 97:2910–2915.
- Ahmad-Annur, A., Tabrizi, S.J., and Fisher, E.M. (2003). Mouse models as a tool for understanding neurodegenerative diseases. *Curr. Opin. Neurol.* 16:451–458.
- Andra, K., Abramowski, D., Duke, M., Probst, A., Wiederholt, K., Burki, K., Goedert, M., Sommer, B., and Staufenbiel, M. (1996). Expression of APP in transgenic mice: a comparison of neuron-specific promoters. *Neurobiol. Aging* 17:183–190.
- Arai, H., Lee, V.-Y., Messinger, M., Greenberg, B., Lowery, D., and Trojanowski, J. (1991). Expression patterns of β -amyloid precursor protein (β -APP) in neural and nonneural tissues from Alzheimer's disease and control subjects. *Ann. Neurol.* 30:686–693.
- Auluck, P.K., Chan, H.Y., Trojanowski, J.Q., Lee, V.M., and Bonini, N.M. (2002). Chaperone suppression of alpha-synuclein toxicity in a *Drosophila* model for Parkinson's disease. *Science* 295:865–868.
- Bales, K.R., Verina, T., Dodel, R.C., Du, Y., Altstiel, L., Bender, M., Hyslop, P., Johnstone, E.M., Little, S.P., Cummins, D.J., Piccardo, P., Ghetti, B., and Paul, S.M. (1997). Lack of apolipoprotein E dramatically reduces amyloid beta-peptide deposition. *Nat. Genet.* 17:263–264.
- Beal, M.F. (2001). Experimental models of Parkinson's disease. *Nat. Rev. Neurosci.* 2:325–334.
- Beaulieu, J.M., Nguyen, M.D., and Julien, J.P. (1999). Late onset of motor neurons in mice overexpressing wild-type peripherin. *J. Cell Biol.* 147:531–544.
- Beaulieu, J.M., Jacomy, H., and Julien, J.P. (2000). Formation of intermediate filament protein aggregates with disparate effects in two transgenic mouse models lacking the neurofilament light subunit. *J. Neurosci.* 20:5321–5328.
- Berger-Sweeney, J., McPhie, D.L., Arters, J.A., Greenan, J., Oster-Granite, M.L., and Neve, R.L. (1999). Impairments in learning and memory accompanied by neurodegeneration in mice transgenic for the carboxyl-terminus of the amyloid precursor protein. *Mol. Brain Res.* 66:150–162.

- Bertoli-Avella, A.M., Oostra, B.A., and Heutink, P. (2004). Chasing genes in Alzheimer's and Parkinson's disease. *Hum. Genet.* 114:413–438.
- Betarbet, R., Sherer, T.B., and Greenamyre, J.T. (2002). Animal models of Parkinson's disease. *Bioessays* 24:308–318.
- Borchelt, D., Thinakaran, G., Eckman, C., Lee, M., Davenport, F., Ratovitsky, T., Prada, C., Kim, G., Seekins, S., and Yager, D. (1996). Familial Alzheimer's disease-linked presenilin 1 variants elevate A β 1–42/1–40 ratio in vitro and in vivo. *Neuron* 17:1005–1013.
- Borchelt, D., Ratovitski, T., van Lare, J., Lee, M., Gonzales, V., Jenkins, N., Copeland, N., Price, D., and Sisodia, S. (1997). Accelerated amyloid deposition in the brains of transgenic mice coexpressing mutant presenilin 1 and amyloid precursor proteins. *Neuron* 19:939–945.
- Bornemann, K.D., and Staufenbiel, M. (2000). Transgenic mouse models of Alzheimer's disease. *Ann. N. Y. Acad. Sci.* 908:260–266.
- Bossy-Wetzel, E., Schwarzenbacher, R., and Lipton, S.A. (2004). Molecular pathways to neurodegeneration. *Nat. Med.* 10(Suppl):S2–S9.
- Boutajangout, A., Leroy, K., Touchet, N., Authélet, M., Blanchard, V., Tremp, G., Pradier, L., and Brion, J.P. (2002). Increased tau phosphorylation but absence of formation of neurofibrillary tangles in mice double transgenic for human tau and Alzheimer mutant (M146L) presenilin-1. *Neurosci. Lett.* 318:29–33.
- Boutajangout, A., Authélet, M., Blanchard, V., Touchet, N., Tremp, G., Pradier, L., and Brion, J.P. (2004). Characterisation of cytoskeletal abnormalities in mice transgenic for wild-type human tau and familial Alzheimer's disease mutants of APP and presenilin-1. *Neurobiol. Dis.* 15:47–60.
- Braak, H., Del Tredici, K., Bratzke, H., Hamm-Clement, J., Sandmann-Keil, D., and Rub, U. (2002). Staging of the intracerebral inclusion body pathology associated with idiopathic Parkinson's disease (preclinical and clinical stages). *J. Neurol.* 249(Suppl 3):III/1–5.
- Brion, J.P. (1998). The role of neurofibrillary tangles in Alzheimer disease. *Acta Neurol. Belg.* 98:165–174.
- Brion, J.-P., Tremp, G., and Octave, J.-N. (1999). Transgenic expression of the shortest human Tau affects its compartmentalization and its phosphorylation as in the pretangle stage of Alzheimer's disease. *Am. J. Pathol.* 154:255–270.
- Brion, J.P., Anderton, B.H., Authélet, M., Dayanandan, R., Leroy, K., Lovestone, S., Octave, J.N., Pradier, L., Touchet, N., and Tremp, G. (2001). Neurofibrillary tangles and tau phosphorylation. *Biochem. Soc. Symp.* 67:81–88.
- Brujin, L., Houseweart, M., Kato, S., Anderson, K., Anderson, S., Ohama, E., Reaume, A., Scott, R., and Cleveland, D. (1998). Aggregation and motor neuron toxicity of an ALS-linked SOD1 mutant independent from wild-type SOD1. *Science* 281:1851–1854.
- Brujin, L.I., Becher, M.W., Lee, M.K., Anderson, K.L., Jenkins, N.A., Copeland, N.G., Sisodia, S.S., Rothstein, J.D., Borchelt, D.R., Price, D.L., and Cleveland, D.W. (1997). ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. *Neuron* 18:327–338.
- Burright, E.N., Clark, H.B., Servadio, A., Matilla, T., Feddersen, R.M., Yunis, W.S., Duvick, L.A., Zoghbi, H.Y., and Orr, H.T. (1995). SCA1 transgenic mice: a model for neurodegeneration caused by an expanded CAG trinucleotide repeat. *Cell* 82:937–948.
- Buttini, M., Yu, G.Q., Shockley, K., Huang, Y., Jones, B., Masliah, E., Mallory, M., Yeo, T., Longo, F.M., and Mucke, L. (2002). Modulation of Alzheimer-like synaptic and cholinergic deficits in transgenic mice by human apolipoprotein E depends on isoform, aging, and overexpression of amyloid beta peptides but not on plaque formation. *J. Neurosci.* 22:10539–10548.
- Campbell, I.L. (1998). Structural and functional impact of the transgenic expression of cytokines in the CNS. *Ann. N.Y. Acad. Sci.* 840:83–96.
- Campbell, I.L., Stalder, A.K., Akwa, Y., Pagenstecher, A., and Asensio, V.C. (1998). Transgenic models to study the actions of cytokines in the central nervous system. *Neuroimmunomodulation* 5:126–135.
- Carlson, G., Borchelt, D., Dake, A., Turner, S., Danielson, V., Coffin, J., Eckman, C., Meiners, J., Nilsen, S., Younkin, S., and Hsiao, K. (1997). Genetic modifications of the phenotypes produced by amyloid precursor protein overexpression in transgenic mice. *Hum. Mol. Genet.* 6:1951–1959.
- Carter, R.J., Lione, L.A., Humby, T., Mangiarini, L., Mahal, A., Bates, G.P., Dunnett, S.B., and Morton, A.J. (1999). Characterization of progressive motor deficits in mice transgenic for the human Huntington's disease mutation. *J. Neurosci.* 19:3248–3257.
- Cemal, C.K., Huxley, C., and Chamberlain, S. (1999). Insertion of expanded CAG trinucleotide repeat motifs into a yeast artificial chromosome containing the human Machado-Joseph disease gene. *Gene* 236:53–61.
- Cemal, C.K., Carroll, C.J., Lawrence, L., Lowrie, M.B., Ruddle, P., Al-Mahdawi, S., King, R.H., Pook, M.A., Huxley, C., and Chamberlain, S. (2002). YAC transgenic mice carrying pathological alleles of the MJD1 locus exhibit a mild and slowly progressive cerebellar deficit. *Hum. Mol. Genet.* 11:1075–1094.

- Chambers, D.M., Peters, J., and Abbott, C.M. (1998). The lethal mutation of the mouse wasted (*wst*) is a deletion that abolishes expression of a tissue-specific isoform of translation elongation factor 1 α , encoded by the *Eef1a2* gene. *Proc. Natl. Acad. Sci. USA* 95:4463–4468.
- Chapman, P.F., White, G.L., Jones, M.W., Cooper-Blacketer, D., Marshall, V.J., Irizarry, M., Younkin, L., Good, M.A., Bliss, T.V., Hyman, B.T., Younkin, S.G., and Hsiao, K.K. (1999). Impaired synaptic plasticity and learning in aged amyloid precursor protein transgenic mice. *Nat. Neurosci.* 2:271–276.
- Cheng, I.H., Palop, J.J., Esposito, L.A., Bien-Ly, N., Yan, F., and Mucke, L. (2004). Aggressive amyloidosis in mice expressing human amyloid peptides with the Arctic mutation. *Nat. Med.* 10:1190–1192.
- Chishti, M.A., Yang, D.S., Janus, C., Phinney, A.L., Horne, P., Pearson, J., Strome, R., Zuker, N., Loukides, J., French, J., Turner, S., Lozza, G., Grilli, M., Kunicki, S., Morissette, C., Paquette, J., Gervais, F., Bergeron, C., Fraser, P.E., Carlson, G.A., George-Hyslop, P.S., and Westaway, D. (2001). Early-onset amyloid deposition and cognitive deficits in transgenic mice expressing a double mutant form of amyloid precursor protein 695. *J. Biol. Chem.* 276:21562–21570.
- Citron, M., Westaway, D., Xia, W., Carlson, G., Diehl, T., Levesque, G., Johnson-Wood, K., Lee, M., Subert, P., Davis, A., Kholodenko, D., Motter, R., Sherrington, R., Perry, B., Hong, Y., Strome, R., Lieberburg, I., Rommens, J., Kim, S., Schenk, D., Fraser, P., St. George Hyslop, P., and Selkoe, D. (1997). Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid beta-protein in both transfected cells and transgenic mice. *Nat. Med.* 3:67–72.
- Clark, H.B., and Orr, H.T. (2000). Spinocerebellar ataxia type 1—modeling the pathogenesis of a polyglutamine neurodegenerative disorder in transgenic mice. *J. Neuropathol. Exp. Neurol.* 59:265–270.
- Clark, R., and Goate, A. (1993). Molecular genetics of Alzheimer's disease. *Arch. Neurol.* 50:1164–1172.
- Cole, G., Masliah, E., Huynh, T., DeTeresa, R., Terry, R., Okudea, C., and Saitoh, T. (1989). An antiserum against amyloid β -protein precursor detects a unique peptide in Alzheimer brain. *Neurosci. Lett.* 100:340–346.
- Conway, K., Harper, J., and Lansbury, P. (1998). Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. *Nat. Med.* 4:1318–1320.
- Conway, K.A., Lee, S.J., Rochet, J.C., Ding, T.T., Williamson, R.E., and Lansbury, P.T., Jr. (2000). Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy. *Proc. Natl. Acad. Sci. USA* 97:571–576.
- Conway, K.A., Rochet, J.C., Bieganski, R.M., and Lansbury, P.T., Jr. (2001). Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct. *Science* 294:1346–1349.
- Cossee, M., Puccio, H., Gansmuller, A., Koutnikova, H., Dierich, A., LeMeur, M., Fischbeck, K., Dolle, P., and Koenig, M. (2000). Inactivation of the Friedreich ataxia mouse gene leads to early embryonic lethality without iron accumulation. *Hum. Mol. Genet.* 9:1219–1226.
- Cote, F., Collard, J.F., and Julien, J.P. (1993). Progressive neuronopathy in transgenic mice expressing the human neurofilament heavy gene: a mouse model of amyotrophic lateral sclerosis. *Cell* 73:35–46.
- Cras, P., Kawai, M., Lowery, D., Gonzalez-DeWhitt, P., Greenberg, B., and Perry, G. (1991). Senile plaque neurites in Alzheimer disease accumulate amyloid precursor protein. *Proc. Natl. Acad. Sci. USA* 88:7552–7556.
- Cruts, M., and Van Broeckhoven, C. (1998). Molecular genetics of Alzheimer's disease. *Ann. Med.* 30:560–565.
- Cummings, C.J., and Zoghbi, H.Y. (2000). Trinucleotide repeats: mechanisms and pathophysiology. *Annu. Rev. Genom. Hum. Genet.* 1:281–328.
- Dal Canto, M.C., and Gurney, M.E. (1995). Neuropathological changes in two lines of mice carrying a transgene for mutant human Cu,Zn SOD, and in mice overexpressing wild type human SOD: a model of familial amyotrophic lateral sclerosis (FALS). *Brain Res.* 676:25–40.
- Davidson, W., Jonas, A., Clayton, D., and George, J. (1998). Stabilization of alpha-synuclein secondary structure upon binding to synthetic membranes. *J. Biol. Chem.* 273:9443–9449.
- Dawson, T., Mandir, A., and Lee, M. (2002). Animal models of PD: pieces of the same puzzle? *Neuron* 35:219–222.
- Dawson, T.M., and Dawson, V.L. (2003). Molecular pathways of neurodegeneration in Parkinson's disease. *Science* 302:819–822.
- de Almeida, L.P., Ross, C.A., Zala, D., Aebischer, P., and Deglon, N. (2002). Lentiviral-mediated delivery of mutant huntingtin in the striatum of rats induces a selective neuropathology modulated by polyglutamine repeat size, huntingtin expression levels, and protein length. *J. Neurosci.* 22:3473–3483.
- Deng, H.X., Hentati, A., Tainer, J.A., Iqbal, Z., Cayabyab, A., Hung, W.Y., Getzoff, E.D., Hu, P., Herzfeldt, B., and Roos, R.P. (1993). Amyotrophic lateral sclerosis and structural defects in Cu,Zn superoxide dismutase. *Science* 261:1047–1051.

- Di Monte, D.A. (2003). The environment and Parkinson's disease: is the nigrostriatal system preferentially targeted by neurotoxins? *Lancet Neurol.* 2:531–538.
- Dickson, D., Farlo, J., Davies, P., Crystal, H., Fuld, P., and Yen, S. (1988). Alzheimer disease. A double immunohistochemical study of senile plaques. *Am. J. Pathol.* 132:86–101.
- Dickson, D.W., Feany, M.B., Yen, S.H., Mattiace, L.A., and Davies, P. (1996). Cytoskeletal pathology in non-Alzheimer degenerative dementia: new lesions in diffuse Lewy body disease, Pick's disease, and corticobasal degeneration. *J. Neural Transm. Suppl.* 47:31–46.
- Driscoll, M., and Gerstbrein, B. (2003). Dying for a cause: invertebrate genetics takes on human neurodegeneration. *Nat. Rev. Genet.* 4:181–194.
- Drolet, R.E., Behrouz, B., Lookingland, K.J., and Goudreau, J.L. (2004). Mice lacking alpha-synuclein have an attenuated loss of striatal dopamine following prolonged chronic MPTP administration. *Neurotoxicology* 25:761–769.
- Duff, K. (1997). Alzheimer transgenic mouse models come of age. *Trends Neurosci.* 20:279–280.
- Duff, K., Eckman, C., Zehr, C., Yu, X., Prada, C., Perez-Tur, J., Hutton, M., Buee, L., Harigaya, Y., Yager, D., Morgan, D., Gordon, M., Holcomb, L., Refolo, L., Zenk, B., Hardy, J., and Younkin, S. (1996). Increased amyloid-beta42(43) in brains of mice expressing mutant presenilin 1. *Nature* 383:710–713.
- Duff, K., Knight, H., Refolo, L.M., Sanders, S., Yu, X., Picciano, M., Malester, B., Hutton, M., Adamson, J., Goedert, M., Burki, K., and Davies, P. (2000). Characterization of pathology in transgenic mice over-expressing human genomic and cDNA tau transgenes. *Neurobiol. Dis.* 7:87–98.
- Feany, M., and Bender, W. (2000). A *Drosophila* model of Parkinson's disease. *Nature* 404:394–398.
- Fernagut, P.O., and Chesselet, M.F. (2004). Alpha-synuclein and transgenic mouse models. *Neurobiol. Dis.* 17:123–130.
- Ferrigno, P., and Silver, P. (2000). Polyglutamine expansions: proteolysis, chaperones, and the dangers of promiscuity. *Neuron* 26:9–12.
- Friedlander, R.M., Brown, R.H., Gagliardini, V., Wang, J., and Yuan, J. (1997). Inhibition of ICE slows ALS in mice. *Nature* 388:31.
- Fryer, J.D., Taylor, J.W., DeMattos, R.B., Bales, K.R., Paul, S.M., Parsadanian, M., and Holtzman, D.M. (2003). Apolipoprotein E markedly facilitates age-dependent cerebral amyloid angiopathy and spontaneous hemorrhage in amyloid precursor protein transgenic mice. *J. Neurosci.* 23:7889–7896.
- Fujiwara, H., Hasegawa, M., Dohmae, N., Kawashima, A., Masliah, E., Goldberg, M.S., Shen, J., Takio, K., and Iwatsubo, T. (2002). alpha-Synuclein is phosphorylated in synucleinopathy lesions. *Nat. Cell Biol.* 4:160–164.
- Galasko, D., Hansen, L., Katzman, R., Wiederholt, W., Masliah, E., Terry, R., Hill, L., Lessin, P., and Thal, L. (1994). Clinical-neuropathological correlations in Alzheimer's disease and related dementias. *Arch. Neurol.* 51:888–895.
- Games, D., Adams, D., Alessandrini, R., Barbour, R., Berthelette, P., Blackwell, C., Carr, T., Clemes, J., Donaldson, T., Gillespie, F., Guido, T., Hagopian, S., Johnson-Wood, K., Khan, K., Lee, M., Leibowitz, P., Lieberburg, I., Little, S., Masliah, E., McConlogue, L., Montoya-Zavala, M., Mucke, L., Paganini, L., Penniman, E., Power, M., Schenk, D., Seubert, P., Snyder, B., Soriano, F., Tan, H., Vitale, J., Wadsworth, S., Wolozin, B., and Zhao, J. (1995). Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. *Nature* 373:523–527.
- Games, D., Masliah, E., Lee, M., Johnson-Wood, K., and Schenk, D. (1997). Neurodegenerative Alzheimer-like pathology in PDAPP 717V → F transgenic mice. In: Hyman, B., Duyckaerts, C., and Christen, Y., editors. *Connections, cognition and Alzheimer's disease*. Berlin: Springer-Verlag, pp. 105–119.
- Ghiso, J., and Frangione, B. (2001). Cerebral amyloidosis, amyloid angiopathy, and their relationship to stroke and dementia. *J. Alzheimers Dis.* 3:65–73.
- Giasson, B.I., Duda, J.E., Murray, I.V., Chen, Q., Souza, J.M., Hurtig, H.I., Ischiropoulos, H., Trojanowski, J.Q., and Lee, V.M. (2000). Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science* 290:985–989.
- Giasson, B.I., Duda, J.E., Quinn, S.M., Zhang, B., Trojanowski, J.Q., and Lee, V.M. (2002). Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. *Neuron* 34:521–533.
- Goate, A., Chartier-Harlin, M.-C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Guiffra, L., Haynes, A., Irving, N., James, L., Mant, R., Newton, P., Rooke, K., Roques, P., Talbot, C., Williamson, R., Rossor, M., Owen, M., and Hardy, J. (1991). Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* 349:704.
- Goedert, M. (2001). Alpha-synuclein and neurodegenerative diseases. *Nat. Rev. Neurosci.* 2:492–501.
- Gotz, J. (2001). Tau and transgenic animal models. *Brain Res. Brain Res. Rev.* 35:266–286.
- Gotz, J., Chen, F., van Dorpe, J., and Nitsch, R.M. (2001a). Formation of neurofibrillary tangles in P301 tau transgenic mice induced by Abeta 42 fibrils. *Science* 293:1491–1495.

- Gotz, J., Chen, F., Barmettler, R., and Nitsch, R.M. (2001b). Tau filament formation in transgenic mice expressing P301L tau. *J. Biol. Chem.* 276:529–534.
- Gotz, J., Tolnay, M., Barmettler, R., Chen, F., Probst, A., and Nitsch, R.M. (2001c). Oligodendroglial tau filament formation in transgenic mice expressing G272V tau. *Eur. J. Neurosci.* 13:2131–2140.
- Gotz, J., Streffer, J.R., David, D., Schild, A., Hoernndli, F., Pennanen, L., Kurosinski, P., and Chen, F. (2004). Transgenic animal models of Alzheimer's disease and related disorders: histopathology, behavior and therapy. *Mol. Psychiatry* 9:664–683.
- Greenamyre, J.T., Betarbet, R., and Sherer, T.B. (2003). The rotenone model of Parkinson's disease: genes, environment and mitochondria. *Parkinsonism Relat. Disord.* 9(Suppl. 2):S59–S64.
- Grunblatt, E., Mandel, S., and Youdim, M.B. (2000). MPTP and 6-hydroxydopamine-induced neurodegeneration as models for Parkinson's disease: neuroprotective strategies. *J. Neurol.* 247(Suppl. 2):95–102.
- Gurney, M.E., Pu, H., Chiu, A.Y., Dal Canto, M.C., Polchow, C.Y., Alexander, D.D., Caliendo, J., Hentati, A., Kwon, Y. W., and Deng, H.X. (1994). Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* 264:1772–1775.
- Haass, C., and Steiner, H. (2001). Protofibrils, the unifying toxic molecule of neurodegenerative disorders? *Nat. Neurosci.* 4:859–860.
- Hansen, L., Salmon, D., Galasko, D., Masliah, E., Katzman, R., DeTeresa, R., Thal, L., Pay, M., Hofstetter, R., and Klauber, M. (1990). The Lewy body variant of Alzheimer's disease: a clinical and pathologic entity. *Neurology* 40:1–7.
- Hansen, L.A., and Galasko, D. (1992). Lewy body disease. *Curr. Opin. Neurol. Neurosurg.* 5:889–894.
- Hardy, J. (1997). Amyloid, the presenilins and Alzheimer's disease. *Trends Neurosci.* 20:154–159.
- Hardy, J., and Gwinn-Hardy, K. (1998). Genetic classification of primary neurodegenerative disease. *Science* 282:1075–1079.
- Hashimoto, M., and Masliah, E. (1999). Alpha-synuclein in Lewy body disease and Alzheimer's disease. *Brain Pathol.* 9:707–720.
- Hashimoto, M., Hernandez-Ruiz, S., Hsu, L., Sisk, A., Xia, Y., Takeda, A., Sundsmo, M., and Masliah, E. (1998). Human recombinant NACP/ α -synuclein is aggregated and fibrillated in vitro: Relevance for Lewy body disease. *Brain Res.* 799:301–306.
- Hashimoto, M., Takeda, A., Hsu, L.J., Takenouchi, T., and Masliah, E. (1999a). Role of cytochrome *c* as a stimulator of α -synuclein aggregation in Lewy body disease. *J. Biol. Chem.* 274:28849–28852.
- Hashimoto, M., Hsu, L., Xia, Y., Takeda, A., Sundsmo, M., and Masliah, E. (1999b). Oxidative stress induces amyloid-like aggregate formation of NACP/ α -synuclein in vitro. *Neuroreport* 10:717–721.
- Hashimoto, M., Rockenstein, E., Mante, M., Mallory, M., and Masliah, E. (2001). β -Synuclein inhibits alpha-synuclein aggregation: a possible role as an anti-parkinsonian factor. *Neuron* 32:213–223.
- Hashimoto, M., Rockenstein, E., and Masliah, E. (2003). Transgenic models of alpha-synuclein pathology: past, present, and future. *Ann. N. Y. Acad. Sci.* 991:171–188.
- Hashimoto, M., Bar-On, P., Ho, G., Takenouchi, T., Rockenstein, E., Crews, L., and Masliah, E. (2004). Beta-synuclein regulates Akt activity in neuronal cells. A possible mechanism for neuroprotection in Parkinson's disease. *J. Biol. Chem.* 279:23622–23629.
- Hattori, N., Matsumine, H., Asakawa, S., Kitada, T., Yoshino, H., Elibol, B., Brookes, A., Yamamura, Y., Kobayashi, T., Wang, M., Yoritaka, A., Minoshima, S., Shimizu, N., and Mizuno, Y. (1998). Point mutations (Thr240Arg and Ala311Stop) in the *Parkin* gene. *Biochem. Biophys. Res. Commun.* 249:754–758.
- Heintz, N., and Zoghbi, H.Y. (2000). Insights from mouse models into the molecular basis of neurodegeneration. *Annu. Rev. Physiol.* 62:779–802.
- Herzig, M.C., Winkler, D.T., Burgermeister, P., Pfeifer, M., Kohler, E., Schmidt, S.D., Danner, S., Abramowski, D., Sturchler-Pierrat, C., Burki, K., van Duinen, S.G., Maat-Schieman, M.L., Staufenbiel, M., Mathews, P.M., and Jucker, M. (2004). Abeta is targeted to the vasculature in a mouse model of hereditary cerebral hemorrhage with amyloidosis. *Nat. Neurosci.* 7:954–960.
- Higgins, G.A., and Jacobsen, H. (2003). Transgenic mouse models of Alzheimer's disease: phenotype and application. *Behav. Pharmacol.* 14:419–438.
- Hodgson, J.G., Agopyan, N., Gutekunst, C.A., Leavitt, B.R., LePiane, F., Singaraja, R., Smith, D.J., Bissada, N., McCutcheon, K., Nasir, J., Jamot, L., Li, X.J., Stevens, M.E., Rosemond, E., Roder, J.C., Phillips, A.G., Rubin, E.M., Hersch, S.M., and Hayden, M.R. (1999). A YAC mouse model for Huntington's disease with full-length mutant huntingtin, cytoplasmic toxicity, and selective striatal neurodegeneration. *Neuron* 23:181–192.
- Hof, P., and Morrison, J. (1994). The cellular basis of cortical disconnection in Alzheimer disease and related dementing conditions. In: Terry, R., Katzman, R., and Bick, K., editors. *Alzheimer disease*. New York: Raven Press, pp. 197–230.

- Hof, P., Cox, K., and Morrison, J. (1990). Quantitative analysis of a vulnerable subset of pyramidal neurons in Alzheimer's disease: I. Superior frontal and inferior temporal cortex. *J. Comp. Neurol.* 301:44–54.
- Holcomb, L., Gordon, M., McGowan, E., Yu, X., Benkovic, S., Jantzen, P., Wright, K., Saad, I., Mueller, R., Morgan, D., Sanders, P., Zehr, C., O'Campo, K., Hardy, J., Prada, C.-M., Eckman, C., Younkin, S., Hsiao, K., and Duff, K. (1998). Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. *Nat. Med.* 4:97–100.
- Holtzman, D.M., Bales, K.R., Wu, S., Bhat, P., Parsadanian, M., Fagan, A.M., Chang, L.K., Sun, Y., and Paul, S.M. (1999). Expression of human apolipoprotein E reduces amyloid- β deposition in a mouse model of Alzheimer's disease. *J. Clin. Invest.* 103:R15–R21.
- Honer, W.G. (2003). Pathology of presynaptic proteins in Alzheimer's disease: more than simple loss of terminals. *Neurobiol. Aging* 24:1047–1062.
- Hsia, A.Y., Masliah, E., McConlogue, L., Yu, G.-Q., Tatsuno, G., Hu, K., Kholodenko, D., Malenka, R.C., Nicoll, R.A., and Mucke, L. (1999). Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. *Proc. Natl. Acad. Sci. USA* 96:3228–3233.
- Hsiao, K., Chapman, P., Nilsen, S., Eckman, C., Harigaya, Y., Younkin, S., Yang, F., and Cole, G. (1996). Correlative memory deficits, A β elevation, and amyloid plaques in transgenic mice. *Science* 274:99–102.
- Hsu, L.J., Sagara, Y., Arroyo, A., Rockenstein, E., Sisk, A., Mallory, M., Wong, J., Takenouchi, T., Hashimoto, M., and Masliah, E. (2000). α -Synuclein promotes mitochondrial deficiencies and oxidative stress. *Am. J. Pathol.* 157: 401–410.
- Hutton, M., and Hardy, J. (1997). The presenilins and Alzheimer's disease. *Hum. Mol. Genet.* 6:1639–1646.
- Hutton, M., Lendon, C., Rizzu, P., Baker, M., Froelich, S., Houlden, H., Pickering-Brown, S., Chakravarly, S., Isaacs, A., Grover, A., Hackett, J., Adamson, J., Lincoln, S., Dickson, D., Davies, P., Petersen, R., Stevens, M., de Graaff, E., Wauters, E., van Baren, J., Hillebrand, M., Joosse, M., Kwon, J., and Nowotny, P. (1998). Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. *Nature* 393:702–705.
- Hutton, M., Lewis, J., Dickson, D., Yen, S., and McGowan, E. (2001). Analysis of tauopathies with transgenic mice. *Trends Mol. Med.* 7:467–470.
- Hyman, B., VanHoesen, G., Damasio, A., and Barnes, C. (1984). Alzheimer's disease: cell-specific pathology isolates the hippocampal formation. *Science* 225:1168–1170.
- Ikedo, H., Yamaguchi, M., Sugai, S., Aze, Y., Narumiya, S., and Kakizuka, A. (1996). Expanded polyglutamine in the Machado-Joseph disease protein induces cell death in vitro and in vivo. *Nat. Genet.* 13:196–202.
- Irizarry, M., Soriano, F., McNamara, M., Page, K., Schenk, D., Games, D., and Hyman, B. (1997a). Abeta deposition is associated with neuropil changes, but not with overt neuronal loss in the human amyloid precursor protein V717F (PDAPP) transgenic mouse. *J. Neurosci.* 17:7053–7059.
- Irizarry, M.C., McNamara, M., Fedorchak, K., Hsiao, K., and Hyman, B.T. (1997b). APPSw transgenic mice develop age-related A beta deposits and neuropil abnormalities, but no neuronal loss in CA1. *J. Neuropathol. Exp. Neurol.* 56:965–973.
- Irizarry, M., Growdon, W., Gomez-Isla, T., Newell, K., George, J., Clayton, D., and Hyman, B. (1998). Nigral and cortical Lewy bodies and dystrophic nigral neurites in Parkinson's disease and cortical Lewy body disease contain alpha-synuclein immunoreactivity. *J. Neuropathol. Exp. Neurol.* 57:334–337.
- Ishihara, T., Hong, M., Zhang, B., Nakagawa, Y., Lee, M.K., Trojanowski, J.Q., and Lee, V.M. (1999). Age-dependent emergence and progression of a tauopathy in transgenic mice overexpressing the shortest human tau isoform. *Neuron* 24:751–762.
- Ishihara, T., Zhang, B., Higuchi, M., Yoshiyama, Y., Trojanowski, J.Q., and Lee, V.M. (2001a). Age-dependent induction of congophilic neurofibrillary tau inclusions in tau transgenic mice. *Am. J. Pathol.* 158:555–562.
- Ishihara, T., Higuchi, M., Zhang, B., Yoshiyama, Y., Hong, M., Trojanowski, J.Q., and Lee, V.M. (2001b). Attenuated neurodegenerative disease phenotype in tau transgenic mouse lacking neurofilaments. *J. Neurosci.* 21:6026–6035.
- Iwai, A. (2000). Properties of NACP/alpha-synuclein and its role in Alzheimer's disease. *Biochim. Biophys. Acta* 1502:95–109.
- Iwai, A., Masliah, E., Yoshimoto, M., De Silva, R., Ge, N., Kittel, A., and Saitoh, T. (1994). The precursor protein of non-A β component of Alzheimer's disease amyloid (NACP) is a presynaptic protein of the central nervous system. *Neuron* 14:467–475.
- Jackson, C.E., and Bryan, W.W. (1998). Amyotrophic lateral sclerosis. *Semin. Neurol.* 18:27–39.
- Jahroudi, N., Schmaier, A., Srikanth, S., Mahdi, F., Lutka, F.A., and Bowser, R. (2003). Von Willebrand factor promoter targets the expression of amyloid beta protein precursor to brain vascular endothelial cells of transgenic mice. *J. Alzheimers Dis.* 5:149–158.

- Jakes, R., Spillantini, M., and Goedert, M. (1994). Identification of two distinct synucleins from human brain. *FEBS Lett.* 345:27–32.
- Jankowsky, J.L., Slunt, H.H., Ratovitski, T., Jenkins, N.A., Copeland, N.G., and Borchelt, D.R. (2001). Co-expression of multiple transgenes in mouse CNS: a comparison of strategies. *Biomol. Eng.* 17:157–165.
- Jensen, P., Hojrup, P., Hager, H., Nielsen, M., Jacobsen, L., Olesen, O., Gliemann, J., and Jakes, R. (1997). Binding of A β to α - and β -synucleins: identification of segments in α -synuclein/NAC precursor that bind A β and NAC. *Biochem. J.* 323:539–546.
- Jia, T., Liu, Y.E., Liu, J., and Shi, Y.E. (1999). Stimulation of breast cancer invasion and metastasis by synuclein gamma. *Cancer Res.* 59:742–747.
- Jo, E., McLaurin, J., Yip, C., St.George-Hyslop, P., and Graser, P. (2000). Alpha-synuclein membrane interactions and lipid specificity. *J. Biol. Chem.* 275:34328–34334.
- Kahle, P., Neumann, M., Ozmen, L., Muller, V., Jacobsen, H., Schindzielorz, A., Okochi, M., Leimer, U., van der Putten, H., Probst, A., Kremmer, E., Kretzschmar, H., and Haass, C. (2000). Subcellular localization of wild-type and Parkinson's disease-associated mutant alpha-synuclein in human and transgenic mouse brains. *J. Neurosci.* 20:6365–6373.
- Kahle, P.J., Neumann, M., Ozmen, L., Muller, V., Odo, S., Okamoto, N., Jacobsen, H., Iwatsubo, T., Trojanowski, J.Q., Takahashi, H., Wakabayashi, K., Bogdanovic, N., Riederer, P., Kretzschmar, H.A., and Haass, C. (2001). Selective insolubility of alpha-synuclein in human Lewy body diseases is recapitulated in a transgenic mouse model. *Am. J. Pathol.* 159:2215–2225.
- Kahle, P.J., Neumann, M., Ozmen, L., Muller, V., Jacobsen, H., Spooren, W., Fuss, B., Mallon, B., Macklin, W.B., Fujiwara, H., Hasegawa, M., Iwatsubo, T., Kretzschmar, H.A., and Haass, C. (2002). Hyperphosphorylation and insolubility of alpha-synuclein in transgenic mouse oligodendrocytes. *EMBO Rep.* 3:583–588.
- Kins, S., Crameri, A., Evans, D.R., Hemmings, B.A., Nitsch, R.M., and Gotz, J. (2001). Reduced protein phosphatase 2A activity induces hyperphosphorylation and altered compartmentalization of tau in transgenic mice. *J. Biol. Chem.* 276:38193–38200.
- Kirik, D., Rosenblad, C., Burger, C., Lundberg, C., Johansen, T.E., Muzyczka, N., Mandel, R.J., and Bjorklund, A. (2002). Parkinson-like neurodegeneration induced by targeted overexpression of alpha-synuclein in the nigrostriatal system. *J. Neurosci.* 22:2780–2791.
- Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 392:605–608.
- Klein, R.L., King, M.A., Hamby, M.E., and Meyer, E.M. (2002). Dopaminergic cell loss induced by human A30P alpha-synuclein gene transfer to the rat substantia nigra. *Hum. Gene Ther.* 13:605–612.
- Klucken, J., Shin, Y., Masliah, E., Hyman, B.T., and McLean, P.J. (2004). Hsp70 Reduces alpha-synuclein aggregation and toxicity. *J. Biol. Chem.* 279:25497–25502.
- Koo, E., Lansbury, P.J., and Kelly, J. (1999). Amyloid diseases: abnormal protein aggregation in neurodegeneration. *Proc. Natl. Acad. Sci. USA* 96:9989–9990.
- Kosaka, K., Yoshimura, M., Ikeda, K., and Budka, H. (1984). Diffuse type of Lewy body disease. Progressive dementia with abundant cortical Lewy bodies and senile changes of varying degree—a new disease? *Clin. Neuropathol.* 3:183–192.
- Kosik, K.S. (1990). Tau protein and neurodegeneration. *Mol. Neurobiol.* 4:171–179.
- Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J., Schols, L., and Reiss, O. (1998). Ala30Pro mutation in the gene encoding α -synuclein in Parkinson's disease. *Nat. Genet.* 18:106–108.
- Kumar-Singh, S., Dewachter, I., Moechars, D., Lubke, U., De Jonghe, C., Ceuterick, C., Checler, F., Naidu, A., Cordell, B., Cras, P., Van Broeckhoven, C., and Van Leuven, F. (2000). Behavioral disturbances without amyloid deposits in mice overexpressing human amyloid precursor protein with Flemish (A692G) or Dutch (E693Q) mutation. *Neurobiol. Dis.* 7:9–22.
- LaFerla, F., Tinkle, B., Bieberich, C., Haudenschild, C., and Jay, G. (1995). The Alzheimer's A beta peptide induces neurodegeneration and apoptotic cell death in transgenic mice. *Nat. Genet.* 9:21–30.
- Langston, J.W., Langston, E.B., and Irwin, I. (1984). MPTP-induced parkinsonism in human and non-human primates—clinical and experimental aspects. *Acta Neurol. Scand. Suppl.* 100:49–54.
- Lansbury, P.T.J. (1999). Evolution of amyloid: what normal protein folding may tell us about fibrillogenesis and disease. *Proc. Natl. Acad. Sci. USA* 96:3342–3344.
- Lee, M.K., Stirling, W., Xu, Y., Xu, X., Qui, D., Mandir, A.S., Dawson, T.M., Copeland, N.G., Jenkins, N.A., and Price, D.L. (2002). Human alpha-synuclein-harboring familial Parkinson's disease-linked Ala-53 $\rightarrow$ Thr mutation causes neurodegenerative disease with alpha-synuclein aggregation in transgenic mice. *Proc. Natl. Acad. Sci. USA* 99:8968–8973.

- Lee, V.M., Giasson, B.I., and Trojanowski, J.Q. (2004). More than just two peas in a pod: common amyloidogenic properties of tau and alpha-synuclein in neurodegenerative diseases. *Trends Neurosci.* 27:129–134.
- Lewis, J., McGowan, E., Rockwood, J., Melrose, H., Nacharaju, P., Van Slegtenhorst, M., Gwinn-Hardy, K., Paul Murphy, M., Baker, M., Yu, X., Duff, K., Hardy, J., Corral, A., Lin, W.L., Yen, S.H., Dickson, D.W., Davies, P., and Hutton, M. (2000). Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein. *Nat. Genet.* 25:402–405.
- Lewis, J., Dickson, D.W., Lin, W.L., Chisholm, L., Corral, A., Jones, G., Yen, S.H., Sahara, N., Skipper, L., Yager, D., Eckman, C., Hardy, J., Hutton, M., and McGowan, E. (2001). Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. *Science* 293:1487–1491.
- Li, F., Calingasan, N.Y., Yu, F., Mauck, W.M., Toidze, M., Almeida, C.G., Takahashi, R.H., Carlson, G.A., Flint Beal, M., Lin, M.T., and Gouras, G.K. (2004). Increased plaque burden in brains of APP mutant MnSOD heterozygous knockout mice. *J. Neurochem.* 89:1308–1312.
- Litvan, I. (1999). Recent advances in atypical parkinsonian disorders. *Curr. Opin. Neurol.* 12:441–446.
- Lo Bianco, C., Ridet, J.L., Schneider, B.L., Deglon, N., and Aebischer, P. (2002). alpha-Synucleinopathy and selective dopaminergic neuron loss in a rat lentiviral-based model of Parkinson's disease. *Proc. Natl. Acad. Sci. USA* 99: 10813–10818.
- Mandybur, T.I. (1986). Cerebral amyloid angiopathy: the vascular pathology and complications. *J. Neuropathol. Exp. Neurol.* 45:79–90.
- Mangiarini, L., Sathasivam, K., Seller, M., Cozens, B., Harper, A., Hetherington, C., Lawton, M., Trottier, Y., Lehrach, H., Davies, S.W., and Bates, G.P. (1996). Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. *Cell* 87:493–506.
- Margolis, R.L., and Ross, C.A. (2001). Expansion explosion: new clues to the pathogenesis of repeat expansion neurodegenerative diseases. *Trends Mol. Med.* 7:479–482.
- Martin, L., Cork, L., Koo, E., Sisodia, S., Weidemann, A., Beyreuther, K., Masters, C., and Price, D. (1989). Localization of amyloid precursor protein (APP) in brains of young and aged monkeys. *Soc. Neurosci. Abstr.* 15:23.
- Masliah, E. (1995). Mechanisms of synaptic dysfunction in Alzheimer's disease. *Histol. Histopathol.* 10:509–519.
- Masliah, E. (1998). The role of synaptic proteins in neurodegenerative disorders. *Neurosci. News* 1:14–20.
- Masliah, E. (2000). The role of synaptic proteins in Alzheimer's disease. *Ann. N. Y. Acad. Sci.* 924:68–75.
- Masliah, E. (2001). Recent advances in the understanding of the role of synaptic proteins in Alzheimer's disease and other neurodegenerative disorders. *J. Alzheimers Dis.* 3:1–9.
- Masliah, E., and Terry, R. (1993). The role of synaptic proteins in the pathogenesis of disorders of the central nervous system. *Brain Pathol.* 3:77–85.
- Masliah, E., and Terry, R. (1994). The role of synaptic pathology in the mechanisms of dementia in Alzheimer's disease. *Clin. Neurosci.* 1:192–198.
- Masliah, E., and Rockenstein, E. (2000). Genetically altered transgenic models of Alzheimer's disease. *J. Neural Transm. Suppl.* 59:175–183.
- Masliah, E., Mallory, M., Hansen, L., Alford, M., Albright, T., Terry, R., Shapiro, P., Sundsmo, M., and Saitoh, T. (1991). Immunoreactivity of CD45, a protein phosphotyrosine phosphatase, in Alzheimer disease. *Acta Neuropathol.* 83: 12–20.
- Masliah, E., Mallory, M., Hansen, L., Alford, M., DeTeresa, R., and Terry, R. (1993). An antibody against phosphorylated neurofilaments identifies a subset of damaged association axons in Alzheimer's disease. *Am. J. Pathol.* 142:871–882.
- Masliah, E., Iwai, A., Mallory, M., Ueda, K., and Saitoh, T. (1996a). Altered presynaptic protein NACP is associated with plaque formation and neurodegeneration in Alzheimer's disease. *Am. J. Pathol.* 148:201–210.
- Masliah, E., Sisk, A., Mallory, M., Mucke, L., Schenk, D., and Games, D. (1996b). Comparison of neurodegenerative pathology in transgenic mice overexpressing V717F β -amyloid precursor protein and Alzheimer's disease. *J. Neurosci.* 16:5795–5811.
- Masliah, E., Mallory, M., Ge, N., and Saitoh, T. (1992). Amyloid precursor protein is localized in growing neurites of neonatal rat brain. *Brain Res.* 593:323–328.
- Masliah, E., Rockenstein, E., Veinbergs, I., Mallory, M., Hashimoto, M., Takeda, A., Sagara, Y., Sisk, A., and Mucke, L. (2000). Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. *Science* 287:1265–1269.
- Masliah, E., Sisk, A., Mallory, M., and Games, D. (2001a). Neurofibrillary pathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. *J. Neuropathol. Exp. Neurol.* 60:357–368.
- Masliah, E., Rockenstein, E., Veinbergs, I., Sagara, Y., Mallory, M., Hashimoto, M., and Mucke, L. (2001b). β Amyloid peptides enhance α -synuclein accumulation and neuronal deficits in a transgenic mouse model likning Alzheimer's and Parkinson's disease. *Proc. Natl. Acad. Sci. USA* 98:12245–12250.

- Masliah, E., Rockenstein, E., Veinbergs, I., Sagara, Y., Mallory, M., Hashimoto, M., and Mucke, L. (2001c). beta-Amyloid peptides enhance alpha-synuclein accumulation and neuronal deficits in a transgenic mouse model linking Alzheimer's disease and Parkinson's disease. *Proc. Natl. Acad. Sci. USA* 98:12245–12250.
- Masters, C., Multhaup, G., Simms, G., Pottgesser, J., Martins, R., and Beyreuther, K. (1985). Neuronal origin of a cerebral amyloid: neurofibrillary tangles of Alzheimer's disease contain the same protein as the amyloid of plaque cores and blood vessels. *EMBO J.* 4:2757–2763.
- Matsuoka, Y., Vila, M., Lincoln, S., McCormack, A., Picciano, M., LaFrancois, J., Yu, X., Dickson, D., Langston, W.J., McGowan, E., Farrer, M., Hardy, J., Duff, K., Przedborski, S., and Di Monte, D.A. (2001). Lack of nigral pathology in transgenic mice expressing human alpha-synuclein driven by the tyrosine hydroxylase promoter. *Neurobiol. Dis.* 8:535–539.
- McGowan, E., Sanders, S., Iwatsubo, T., Takeuchi, A., Saido, T., Zehr, C., Yu, X., Uljon, S., Wang, R., Mann, D., Dickson, D., and Duff, K. (1999). Amyloid phenotype characterization of transgenic mice overexpressing both mutant amyloid precursor protein and mutant presenilin 1 transgenes. *Neurobiol. Dis.* 6:231–244.
- McInerney-Leo, A., Hadley, D.W., Gwinn-Hardy, K., and Hardy, J. (2005). Genetic testing in Parkinson's disease. *Mov. Disord.* 20:1–10.
- McKeith, I., Galasko, D., Kosaka, K., Perry, E., Dickson, D., Hansen, L., Salmon, D., Lowe, J., Mirra, S., Byrne, E., Quinn, N., Edwardson, J., Ince, P., Bergeron, C., Burns, A., Miller, B., Lovestone, S., Collerton, D., Jansen, E., de Vos, R., Wilcock, G., Jellinger, K., and Perry, R. (1996). Clinical and pathological diagnosis of dementia with Lewy bodies (DLB): report of the CDLB International Workshop. *Neurology* 47:1113–1124.
- McKeith, I.G. (2000). Spectrum of Parkinson's disease, Parkinson's dementia, and Lewy body dementia. *Neurol. Clin.* 18:865–902.
- Meier, J., Couillard-Despres, S., Jacomy, H., Gravel, C., and Julien, J.P. (1999). Extra neurofilament NF-L subunits rescue motor neuron disease caused by overexpression of the human NF-H gene in mice. *J. Neuropathol. Exp. Neurol.* 58:1099–1110.
- Menalled, L.B., and Chesselet, M.F. (2002). Mouse models of Huntington's disease. *Trends Pharmacol. Sci.* 23:32–39.
- Miranda, C.J., Santos, M.M., Ohshima, K., Smith, J., Li, L., Bunting, M., Cossee, M., Koenig, M., Sequeiros, J., Kaplan, J., and Pandolfo, M. (2002). Frataxin knockin mouse. *FEBS Lett.* 512:291–297.
- Moechars, D., Dewachter, I., Lorent, K., Reverse, D., Baekelandt, V., Naidu, A., Tesseur, I., Spittaels, K., van den Haute, C., Checler, F., Godaux, E., Cordell, B., and Van Leuven, F. (1999). Early phenotypic changes in transgenic mice that overexpress different mutants of amyloid precursor protein in brain. *J. Biol. Chem.* 274:6483–6492.
- Morgan, D. (2003). Learning and memory deficits in APP transgenic mouse models of amyloid deposition. *Neurochem. Res.* 28:1029–1034.
- Muchowski, P.J. (2002). Protein misfolding, amyloid formation, and neurodegeneration: a critical role for molecular chaperones? *Neuron* 35:9–12.
- Mucke, L., Oldstone, M.B.A., Morris, J., and Nerenberg, M. (1991). Rapid activation of astrocyte-specific expression of GFAP-lacZ transgene by focal injury. *New Biol.* 3:465–474.
- Mucke, L., Yu, G.Q., McConlogue, L., Rockenstein, E.M., Abraham, C.R., and Masliah, E. (2000a). Astroglial expression of human alpha(1)-antichymotrypsin enhances Alzheimer-like pathology in amyloid protein precursor transgenic mice. *Am. J. Pathol.* 157:2003–2010.
- Mucke, L., Masliah, E., Yu, G.Q., Mallory, M., Rockenstein, E.M., Tatsuno, G., Hu, K., Kholodenko, D., Johnson-Wood, K., and McConlogue, L. (2000b). High-level neuronal expression of abeta 1–42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J. Neurosci.* 20:4050–4058.
- Nakajo, S., Tsukada, K., Omata, K., Nakamura, Y., and Nakaya, K. (1993). A new brain-specific 14-kDa protein is a phosphoprotein. Its complete amino acid sequence and evidence for phosphorylation. *Eur. J. Biochem.* 217:1057–1063.
- Nalbantoglu, J., Tirado-Santiago, G., Lahsaini, A., Poirier, J., Goncalves, O., Verge, G., Momoli, F., Welner, S., Massicotte, G., and Julien, J. (1997). Impaired learning and LTP in mice expressing the carboxy terminus of the Alzheimer amyloid precursor protein. *Nature* 387:500–505.
- Narayanan, V., and Scarlata, S. (2001). Membrane binding and self-association of alpha-synucleins. *Biochemistry* 40:9927–9934.
- Narhi, L., Wood, S.J., Steavenson, S., Jiang, Y., Wu, G.M., Anafi, D., Kaufman, S.A., Martin, F., Sitney, K., Denis, P., Louis, J.C., Wypych, J., Biere, A.L., and Citron, M. (1999). Both familial Parkinson's disease mutations accelerate alpha-synuclein aggregation. *J. Biol. Chem.* 274:9843–9846.
- Newbery, H.J., and Abbott, C.M. (2002). Of mice, men and motor neurons. *Trends Mol. Med.* 8:88–92.
- Nicoll, J.A., Yamada, M., Frackowiak, J., Mazur-Kolecka, B., and Weller, R.O. (2004). Cerebral amyloid angiopathy plays a direct role in the pathogenesis of Alzheimer's disease. Pro-CAA position statement. *Neurobiol. Aging* 25:589–597; discussion 603–584.

- Oddo, S., Caccamo, A., Kitazawa, M., Tseng, B.P., and LaFerla, F.M. (2003a). Amyloid deposition precedes tangle formation in a triple transgenic model of Alzheimer's disease. *Neurobiol. Aging* 24:1063–1070.
- Oddo, S., Caccamo, A., Shepherd, J.D., Murphy, M.P., Golde, T.E., Kaye, R., Metherate, R., Mattson, M.P., Akbari, Y., and LaFerla, F.M. (2003b). Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular A β and synaptic dysfunction. *Neuron* 39:409–421.
- Okochi, M., Walter, J., Koyama, A., Nakajo, S., Baba, M., Iwatsubo, T., Meijer, L., Kahle, P., and Haass, C. (2000). Constitutive phosphorylation of the Parkinson's disease associated α -synuclein. *J. Biol. Chem.* 275:390–397.
- Oosthuysen, B., Moons, L., Storkebaum, E., Beck, H., Nuyens, D., Brusselmans, K., Van Dorpe, J., Hellings, P., Gorselink, M., Heymans, S., Theilmeier, G., Dewerchin, M., Laudenbach, V., Vermeylen, P., Raat, H., Acker, T., Vlemminckx, V., Van Den Bosch, L., Cashman, N., Fujisawa, H., Drost, M.R., Sciort, R., Bruyninckx, F., Hicklin, D.J., Ince, C., Gressens, P., Lupu, F., Plate, K.H., Robberecht, W., Herbert, J.M., Collen, D., and Carmeliet, P. (2001). Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. *Nat. Genet.* 28:131–138.
- Orr, H.T., and Zoghbi, H.Y. (2001). SCA1 molecular genetics: a history of a 13 year collaboration against glutamines. *Hum. Mol. Genet.* 10:2307–2311.
- Orrell, R.W., Habgood, J., Rudge, P., Lane, R.J., and de Belleroche, J.S. (1996). Difficulties in distinguishing sporadic from familial amyotrophic lateral sclerosis. *Ann. Neurol.* 39:810–812.
- Ozawa, K., Tomiyama, T., Maat-Schieman, M.L., Roos, R.A., and Mori, H. (2002). Enhanced A β 40 deposition was associated with increased A β 42–43 in cerebral vasculature with Dutch-type hereditary cerebral hemorrhage with amyloidosis (HCHWA-D). *Ann. N. Y. Acad. Sci.* 977:149–154.
- Paik, S., Lee, J., Kim, D., Chang, C., and Kim, Y. (1998). Self-oligomerization of NACP, the precursor protein of the non-amyloid β 42 protein (A β) component of Alzheimer's disease amyloid, observed in the presence of a C-terminal A β fragment (residues 25–35). *FEBS Lett.* 421:73–76.
- Paik, S.R., Shin, H.J., Lee, J.H., Chang, C.S., and Kim, J. (1999). Copper(II)-induced self-oligomerization of α -synuclein. *Biochem. J.* 340:821–828.
- Palmer, A., and Gershon, S. (1990). Is the neuronal basis of Alzheimer's disease cholinergic or glutamatergic? *FASEB J.* 4:2745–2752.
- Palop, J.J., Jones, B., Kekoni, L., Chin, J., Yu, G.Q., Raber, J., Masliah, E., and Mucke, L. (2003). Neuronal depletion of calcium-dependent proteins in the dentate gyrus is tightly linked to Alzheimer's disease-related cognitive deficits. *Proc. Natl. Acad. Sci. USA* 100:9572–9577.
- Pastor, P., and Goate, A.M. (2004). Molecular genetics of Alzheimer's disease. *Curr. Psychiatr. Rep.* 6:125–133.
- Patrick, G.N., Zuberberg, L., Nikolic, M., de la Monte, S., Dikkes, P., and Tsai, L.H. (1999). Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. *Nature* 402:615–622.
- Perrin, R., Woods, W., Clayton, D., and George, J. (2000). Interaction of human α -synuclein and Parkinson's disease variants with phospholipids: structural analysis using site-directed mutagenesis. *J. Biol. Chem.* 275:34393–34398.
- Perry, E., Perry, R., Blessed, G., and Tomlinson, B. (1977). Neurotransmitter enzyme abnormalities in senile dementia: CAT and GAD activities in necropsy tissue. *J. Neurol. Sci.* 34:247–265.
- Polymopoulos, M., Lavedan, C., Leroy, E., Ide, S., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W., Lazzarini, A., Duvoisin, R., Di Iorio, G., Golbe, L., and Nussbaum, R. (1997). Mutation in the α -synuclein gene identified in families with Parkinson's disease. *Science* 276:2045–2047.
- Poorkaj, P., Bird, T.D., Wijsman, E., Nemens, E., Garruto, R.M., Anderson, L., Andreadis, A., Wiederholt, W.C., Raskind, M., and Schellenberg, G.D. (1998). Tau is a candidate gene for chromosome 17 frontotemporal dementia. *Ann. Neurol.* 43:815–825.
- Price, D.L., Wong, P.C., Markowska, A.L., Lee, M.K., Thinakaran, G., Cleveland, D.W., Sisodia, S.S., and Borchelt, D.R. (2000). The value of transgenic models for the study of neurodegenerative diseases. *Ann. N. Y. Acad. Sci.* 920:179–191.
- Probst, A., Gotz, J., Wiederhold, K.H., Tolnay, M., Mistl, C., Jaton, A.L., Hong, M., Ishihara, T., Lee, V.M., Trojanowski, J.Q., Jakes, R., Crowther, R.A., Spillantini, M.G., Burki, K., and Goedert, M. (2000). Axonopathy and amyotrophy in mice transgenic for human four-repeat tau protein. *Acta Neuropathol. (Berl.)* 99:469–481.
- Prusiner, S., and Scott, M. (1997). Genetics of prions. *Annu. Rev. Genet.* 31:139–175.
- Puccio, H., Simon, D., Cossee, M., Crique-Filipe, P., Tiziano, F., Melki, J., Hindelang, C., Matyas, R., Rustin, P., and Koenig, M. (2001). Mouse models for Friedreich ataxia exhibit cardiomyopathy, sensory nerve defect and Fe-S enzyme deficiency followed by intramitochondrial iron deposits. *Nat. Genet.* 27:181–186.

- Ramassamy, C., Averill, D., Beffert, U., Bastianetto, S., Theroux, L., Lussier-Cacan, S., Cohn, J.S., Christen, Y., Davignon, J., Quirion, R., and Poirier, J. (1999). Oxidative damage and protection by antioxidants in the frontal cortex of Alzheimer's disease is related to the apolipoprotein E genotype. *Free Radic. Biol. Med.* 27:544–553.
- Ranum, L.P., and Day, J.W. (2004). Pathogenic RNA repeats: an expanding role in genetic disease. *Trends Genet.* 20: 506–512.
- Rathke-Hartlieb, S., Kahle, P.J., Neumann, M., Ozmen, L., Haid, S., Okochi, M., Haass, C., and Schulz, J.B. (2001). Sensitivity to MPTP is not increased in Parkinson's disease-associated mutant alpha-synuclein transgenic mice. *J. Neurochem.* 77:1181–1184.
- Reddy, P.H., Williams, M., Charles, V., Garrett, L., Pike-Buchanan, L., Whetsell, W.O., Jr., Miller, G., and Tagle, D.A. (1998). Behavioural abnormalities and selective neuronal loss in HD transgenic mice expressing mutated full-length HD cDNA. *Nat. Genet.* 20:198–202.
- Reddy, P.H., Charles, V., Williams, M., Miller, G., Whetsell, W.O., Jr., and Tagle, D.A. (1999). Transgenic mice expressing mutated full-length HD cDNA: a paradigm for locomotor changes and selective neuronal loss in Huntington's disease. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 354:1035–1045.
- Richfield, E.K., Thiruchelvam, M.J., Cory-Slechta, D.A., Wuertzer, C., Gainetdinov, R.R., Caron, M.G., Di Monte, D.A., and Federoff, H.J. (2002). Behavioral and neurochemical effects of wild-type and mutated human alpha-synuclein in transgenic mice. *Exp. Neurol.* 175:35–48.
- Ripps, M.E., Huntley, G.W., Hof, P.R., Morrison, J.H., and Gordon, J.W. (1995). Transgenic mice expressing an altered murine superoxide dismutase gene provide an animal model of amyotrophic lateral sclerosis. *Proc. Natl. Acad. Sci. USA* 92:689–693.
- Rocchi, A., Pellegrini, S., Siciliano, G., and Murri, L. (2003). Causative and susceptibility genes for Alzheimer's disease: a review. *Brain Res. Bull.* 61:1–24.
- Rochet, J., Conway, K., and Lansbury, P.J. (2000). Inhibition of fibrillization and accumulation of prefibrillar oligomers in mixtures of human and mouse alpha-synuclein. *Biochemistry* 39:10619–10626.
- Rockenstein, E., McConlogue, L., Tan, H., Power, M., Masliah, E., and Mucke, L. (1995). Levels and alternative splicing of amyloid β protein precursor (APP) transcripts in brains of APP transgenic mice and humans with Alzheimer's disease. *J. Biol. Chem.* 270:28257–28267.
- Rockenstein, E., Mallory, M., Mante, M., Sisk, A., and Masliah, E. (2001). Early formation of mature amyloid- β proteins deposits in a mutant APP transgenic model depends on levels of A β 1–42. *J. Neurosci. Res.* 66:573–582.
- Rockenstein, E., Mallory, M., Hashimoto, M., Song, D., Shults, C.W., Lang, I., and Masliah, E. (2002). Differential neuropathological alterations in transgenic mice expressing alpha-synuclein from the platelet-derived growth factor and Thy-1 promoters. *J. Neurosci. Res.* 68:568–578.
- Rogers, J., Luber-Narod, J., Styren, S., and Civin, W. (1988). Expression of immune system-associated antigens by cells of the human central nervous system: relationship to the pathology of Alzheimer's disease. *Neurobiol. Aging* 9: 339–349.
- Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., and Deng, H.X. (1993). Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 362:59–62.
- Ross, C.A. (1995). When more is less: pathogenesis of glutamine repeat neurodegenerative diseases. *Neuron* 15: 493–496.
- Salmon, D., Hansen, L., Masliah, E., Galasko, D., Butters, N., and Katzman, R. (1989). Neuropsychological characteristics of a Lewy body variant of Alzheimer's disease. *Soc. Neurosci. Abstr.* 15:863.
- Scheff, S.W., and Price, D.A. (2003). Synaptic pathology in Alzheimer's disease: a review of ultrastructural studies. *Neurobiol. Aging* 24:1029–1046.
- Schmitz, C., Rutten, B.P., Pielen, A., Schafer, S., Wirths, O., Tremp, G., Czech, C., Blanchard, V., Multhaup, G., Rezaie, P., Korh, H., Steinbusch, H.W., Pradier, L., and Bayer, T.A. (2004). Hippocampal neuron loss exceeds amyloid plaque load in a transgenic mouse model of Alzheimer's disease. *Am. J. Pathol.* 164:1495–1502.
- Selkoe, D. (1989). Amyloid β protein precursor and the pathogenesis of Alzheimer's disease. *Cell* 58:611–612.
- Selkoe, D.J. (1999). Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature* 399(Suppl.): A23–A31.
- Selkoe, D.J., Yamazaki, T., Citron, M., Podlisny, M.B., Koo, E.H., Teplow, D.B., and Haass, C. (1996). The role of APP processing and trafficking pathways in the formation of amyloid beta-protein. *Ann. N. Y. Acad. Sci.* 777:57–64.
- Serpell, L., Berriman, J., Jakes, R., Goedert, M., and Crowther, R. (2000). Fiber diffraction of synthetic α -synuclein filaments shows amyloid-like cross- β conformation. *Proc. Natl. Acad. Sci. USA* 97:4897–4902.
- Shastri, B.S. (2001). Parkinson disease: etiology, pathogenesis and future of gene therapy. *Neurosci. Res.* 41:5–12.

- Shibata, N. (2001). Transgenic mouse model for familial amyotrophic lateral sclerosis with superoxide dismutase-1 mutation. *Neuropathology* 21:82–92.
- Shimura, H., Hattori, N., Kubo, S.-i., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K., and Suzuki, T. (2000). Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat. Genet.* 25:302–305.
- Shulman, J.M., Shulman, L.M., Weiner, W.J., and Feany, M.B. (2003). From fruit fly to bedside: translating lessons from *Drosophila* models of neurodegenerative disease. *Curr. Opin. Neurol.* 16:443–449.
- Siman, R., Reaume, A.G., Savage, M.J., Trusko, S., Lin, Y.G., Scott, R.W., and Flood, D.G. (2000). Presenilin-1 P264L knock-in mutation: differential effects on abeta production, amyloid deposition, and neuronal vulnerability. *J. Neurosci.* 20:8717–8726.
- Singleton, A.B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., Hulihan, M., Peuralinna, T., Dutra, A., Nussbaum, R., Lincoln, S., Crawley, A., Hanson, M., Maraganore, D., Adler, C., Cookson, M.R., Muenster, M., Baptista, M., Miller, D., Blacato, J., Hardy, J., and Gwinn-Hardy, K. (2003). alpha-Synuclein locus triplication causes Parkinson's disease. *Science* 302:841.
- Sisodia, S., Koo, E., Beyreuther, K., Unterbeck, A., and Price, D. (1990). Evidence that beta-amyloid protein in Alzheimer's disease is not derived by normal processing. *Science* 248:492–494.
- Song, D.D., Shults, C.W., Sisk, A., Rockenstein, E., and Masliah, E. (2004). Enhanced substantia nigra mitochondrial pathology in human alpha-synuclein transgenic mice after treatment with MPTP. *Exp. Neurol.* 186:158–172.
- Souza, J., Giasson, B., Chen, Q., Lee, V.-Y., and Ischiropoulos, H. (2000). Dityrosine cross-linking promotes formation of stable alpha-synuclein polymers. *J. Biol. Chem.* 275:18344–18349.
- Spillantini, M., Schmidt, M., Lee, V.-Y., Trojanowski, J., Jakes, R., and Goedert, M. (1997). alpha-Synuclein in Lewy bodies. *Nature* 388:839–840.
- Spillantini, M., Murrell, J., Goedert, M., Farlow, M., Klug, A., and Ghetti, B. (1998). Mutation in the tau gene in familial multiple system tauopathy with presenile dementia. *Proc. Natl. Acad. Sci. USA* 95:7737–7741.
- Spittaels, K., Van den Haute, C., Van Dorpe, J., Bruynseels, K., Vandezande, K., Laenen, I., Geerts, H., Mercken, M., Sciort, R., Van Lommel, A., Loos, R., and Van Leuven, F. (1999). Prominent axonopathy in the brain and spinal cord of transgenic mice overexpressing four-repeat human tau protein. *Am. J. Pathol.* 155:2153–2165.
- Sturchler-Pierrat, C., Abramowski, D., Duke, M., Wiederholt, K., Mistl, C., Rothacher, S., Ledermann, B., Burki, K., Frey, P., Paganetti, P., Waridel, C., Calhoun, M., Jucker, M., Probst, A., Staufenbiel, M., and Sommer, B. (1997). Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology. *Proc. Natl. Acad. Sci. USA* 94:13287–13292.
- Surguchov, A., Surgucheva, I., Solessio, E., and Baehr, W. (1999). Synoretin—a new protein belonging to the synuclein family. *Mol. Cell. Neurosci.* 13:95–103.
- Takeda, A., Mallory, M., Sundsmo, M., Honer, W., Hansen, L., and Masliah, E. (1998). Abnormal accumulation of NACP/alpha-synuclein in neurodegenerative disorders. *Am. J. Pathol.* 152:367–372.
- Tanemura, K., Akagi, T., Murayama, M., Kikuchi, N., Murayama, O., Hashikawa, T., Yoshiike, Y., Park, J.M., Matsuda, K., Nakao, S., Sun, X., Sato, S., Yamaguchi, H., and Takashima, A. (2001). Formation of filamentous tau aggregations in transgenic mice expressing V337M human tau. *Neurobiol. Dis.* 8:1036–1045.
- Tanemura, K., Murayama, M., Akagi, T., Hashikawa, T., Tominaga, T., Ichikawa, M., Yamaguchi, H., and Takashima, A. (2002). Neurodegeneration with tau accumulation in a transgenic mouse expressing V337M human tau. *J. Neurosci.* 22:133–141.
- Tatebayashi, Y., Miyasaka, T., Chui, D.H., Akagi, T., Mishima, K., Iwasaki, K., Fujiwara, M., Tanemura, K., Murayama, M., Ishiguro, K., Planel, E., Sato, S., Hashikawa, T., and Takashima, A. (2002). Tau filament formation and associative memory deficit in aged mice expressing mutant (R406W) human tau. *Proc. Natl. Acad. Sci. USA* 99:13896–13901.
- Terry, R., Hansen, L., and Masliah, E. (1994). Structural basis of the cognitive alterations in Alzheimer disease. In: Terry, R. and Katzman, R., editors. *Alzheimer disease*. New York: Raven Press, pp. 179–196.
- Tremblay, P., Meiner, Z., Galou, M., Heinrich, C., Petromilli, C., Lisse, T., Cayetano, J., Torchia, M., Mobley, W., Bujard, H., DeArmond, S.J., and Prusiner, S.B. (1998). Doxycycline control of prion protein transgene expression modulates prion disease in mice. *Proc. Natl. Acad. Sci. USA* 95:12580–12585.
- Trojanowski, J., and Lee, V. (1998). Aggregation of neurofilament and alpha-synuclein proteins in Lewy bodies: implications for pathogenesis of Parkinson disease and Lewy body dementia. *Arch. Neurol.* 55:151–152.
- Trojanowski, J., Schmidt, M., Shin, R.-W., Bramblett, G., Rao, D., and Lee, V.-Y. (1993). Altered Tau and neurofilament proteins in neurodegenerative diseases: diagnostic implications for Alzheimer's disease and Lewy body dementias. *Brain Pathol.* 3:45–54.

- Trojanowski, J.Q., and Lee, V.M. (2000). "Fatal attractions" of proteins. A comprehensive hypothetical mechanism underlying Alzheimer's disease and other neurodegenerative disorders. *Ann. N. Y. Acad. Sci.* 924:62–67.
- Trojanowski, J.Q., Ishihara, T., Higuchi, M., Yoshiyama, Y., Hong, M., Zhang, B., Forman, M.S., Zhukareva, V., and Lee, V.M. (2002). Amyotrophic lateral sclerosis/parkinsonism dementia complex: transgenic mice provide insights into mechanisms underlying a common tauopathy in an ethnic minority on Guam. *Exp. Neurol.* 176: 1–11.
- Ueda, K., Masliah, E., Xia, Y., Iwai, A., Yoshimoto, M., and Saitoh, T. (1993). Novel amyloid component (NAC) differentiates Alzheimer's disease from normal aging plaques. *Soc. Neurosci. Abstr.* 19:1254.
- Uversky, V.N., and Fink, A.L. (2002). Amino acid determinants of alpha-synuclein aggregation: putting together pieces of the puzzle. *FEBS Lett.* 522:9–13.
- Uversky, V.N., Li, J., Souillac, P., Millett, I.S., Doniach, S., Jakes, R., Goedert, M., and Fink, A.L. (2002). Biophysical properties of the synucleins and their propensities to fibrillate: inhibition of alpha-synuclein assembly by beta- and gamma-synucleins. *J. Biol. Chem.* 277:11970–11978.
- Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A.R., Healy, D.G., Albanese, A., Nussbaum, R., Gonzalez-Maldonado, R., Deller, T., Salvi, S., Cortelli, P., Gilks, W.P., Latchman, D.S., Harvey, R.J., Dallapiccola, B., Auburger, G., and Wood, N.W. (2004). Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* 304:1158–1160.
- van der Putten, H., Wiederhold, K.H., Probst, A., Barbieri, S., Mistl, C., Danner, S., Kauffmann, S., Hofele, K., Spooren, W.P., Ruegg, M.A., Lin, S., Caroni, P., Sommer, B., Tolnay, M., and Bilbe, G. (2000). Neuropathology in mice expressing human alpha-synuclein. *J. Neurosci.* 20:6021–6029.
- van Leeuwen, F., de Kleijn, D., van den Hurk, H., Neubauer, A., Sonnemans, M., Sluijs, J., Keoyceau, S., Ramdjielal, R., Salehi, A., Martens, G., Grosveld, F., Peter, J., Burbach, H., and Hol, E. (1998). Frameshift mutants of beta amyloid precursor protein and ubiquitin-B in Alzheimer's and Down patients. *Science* 279:242–247.
- Van Uden, E., Sagara, Y., Van Uden, J., Orlando, R., Mallory, M., Rockenstein, E., and Masliah, E. (2000). A protective role of the low density lipoprotein receptor-related protein against amyloid β -protein toxicity. *J. Biol. Chem.* 275: 20525–30530.
- Van Uden, E., Mallory, M., Veinbergs, I., Alford, M., Rockenstein, E., and Masliah, E. (2002). Increased extracellular amyloid deposition and neurodegeneration in human amyloid precursor protein transgenic mice deficient in receptor-associated protein. *J. Neurosci.* 22:9298–9304.
- Verma, I.M., and Gage, F.H. (2000). Genome and gene therapy. *Mol. Ther.* 2:95.
- Vila, M., Vukosavic, S., Jackson-Lewis, V., Neystat, M., Jakowec, M., and Przedborski, S. (2000). Alpha-synuclein up-regulation in substantia nigra dopaminergic neurons following administration of the parkinsonian toxin MPTP. *J. Neurochem.* 74:721–729.
- Volles, M.J., and Lansbury, P.T., Jr. (2002). Vesicle permeabilization by protofibrillar alpha-synuclein is sensitive to Parkinson's disease-linked mutations and occurs by a pore-like mechanism. *Biochemistry* 41:4595–4602.
- Volles, M.J., Lee, S.J., Rochet, J.C., Shtilerman, M.D., Ding, T.T., Kessler, J.C., and Lansbury, P.T., Jr. (2001). Vesicle permeabilization by protofibrillar alpha-synuclein: implications for the pathogenesis and treatment of Parkinson's disease. *Biochemistry* 40:7812–7819.
- Wakabayashi, K., Matsumoto, K., Takayama, K., Yoshimoto, M., and Takahashi, H. (1997). NACP, a presynaptic protein, immunoreactivity in Lewy bodies in Parkinson's disease. *Neurosci. Lett.* 239:45–48.
- Wallen, A., and Perlmann, T. (2003). Transcriptional control of dopamine neuron development. *Ann. N. Y. Acad. Sci.* 991:48–60.
- Walsh, D., Tseng, B., Rydel, R., Podlisny, M., and Selkoe, D. (2000). The oligomerization of amyloid beta-protein begins intracellularly in cells derived from human brain. *Biochemistry* 39:10831–10839.
- Wilcock, G., Esiri, M., Bowen, D., and Hughes, A. (1988). The differential involvement of subcortical nuclei in senile dementia of Alzheimer's type. *J. Neurol. Neurosurg. Psychol.* 51:842–849.
- Wong, P.C., Pardo, C.A., Borchelt, D.R., Lee, M.K., Copeland, N.G., Jenkins, N.A., Sisodia, S.S., Cleveland, D.W., and Price, D.L. (1995). An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron* 14:1105–1116.
- Wood, J.D., Beaujeux, T.P., and Shaw, P.J. (2003). Protein aggregation in motor neurone disorders. *Neuropathol. Appl. Neurobiol.* 29:529–545.
- Wood, S.J., Wypych, J., Steavenson, S., Louis, J.C., Citron, M., and Biere, A.L. (1999). alpha-Synuclein fibrillogenesis is nucleation dependent. Implications for the pathogenesis of Parkinson's disease. *J. Biol. Chem.* 274: 19509–19512.
- Wullner, U. (2003). Genes implicated in the pathogenesis of spinocerebellar ataxias. *Drugs Today (Barc.)* 39:927–937.

- Wyss-Coray, T., Masliah, E., Mallory, M., McConlogue, L., Johnson-Wood, K., Campagno, F., Lin, C., Samuels, I., and Mucke, L. (1997). Amyloidogenic role of transforming growth factor β 1 in transgenic mice and Alzheimer's disease. *Nature* 389:603–606.
- Wyss-Coray, T., Lin, C., Yan, F., Yu, G.Q., Rohde, M., McConlogue, L., Masliah, E., and Mucke, L. (2001). TGF-beta1 promotes microglial amyloid-beta clearance and reduces plaque burden in transgenic mice. *Nat. Med.* 7:612–618.
- Wyss-Coray, T., Yan, F., Lin, A.H., Lambris, J.D., Alexander, J.J., Quigg, R.J., and Masliah, E. (2002). Prominent neurodegeneration and increased plaque formation in complement-inhibited Alzheimer's mice. *Proc. Natl. Acad. Sci. USA* 99:10837–10842.
- Xu, Z., Cork, L.C., Griffin, J.W., and Cleveland, D.W. (1993). Increased expression of neurofilament subunit NF-L produces morphological alterations that resemble the pathology of human motor neuron disease. *Cell* 73:23–33.
- Yamamoto, A., Lucas, J.J., and Hen, R. (2000). Reversal of neuropathology and motor dysfunction in a conditional model of Huntington's disease. *Cell* 101:57–66.
- Yoshimoto, M., Iwai, A., Kang, D., Otero, D., Xia, Y., and Saitoh, T. (1995). NACP, the precursor protein of non-amyloid β /A4 protein (A β) component of Alzheimer disease amyloid, binds A β and stimulates A β aggregation. *Proc. Natl. Acad. Sci. USA* 92:9141–9145.
- Yvert, G., Lindenberg, K.S., Picaud, S., Landwehrmeyer, G.B., Sahel, J.A., and Mandel, J.L. (2000). Expanded polyglutamines induce neurodegeneration and trans-neuronal alterations in cerebellum and retina of SCA7 transgenic mice. *Hum. Mol. Genet.* 9:2491–2506.

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