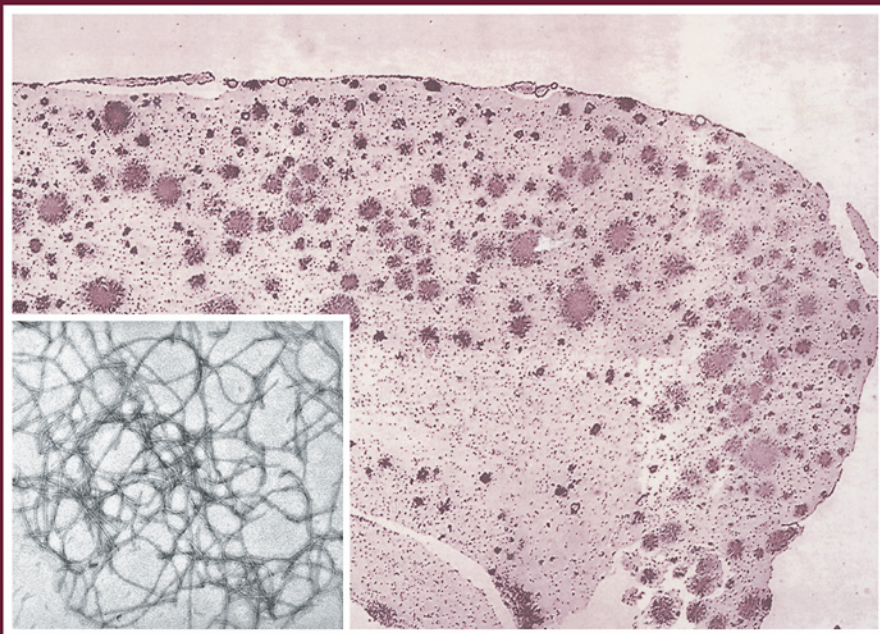


Molecular Biology of Alzheimer's Disease

Genes and Mechanisms Involved in
Amyloid Generation

Edited by

Christian Haass



harwood academic publishers

Also available as a printed book
see title verso for ISBN details

MOLECULAR BIOLOGY OF ALZHEIMER'S DISEASE

MOLECULAR BIOLOGY OF ALZHEIMER'S DISEASE

Genes and Mechanisms Involved in Amyloid Generation

Edited by

CHRISTIAN HAASS
*Central Institute of Mental Health
University of Mannheim
Germany*



harwood academic publishers

Australia • Canada • China • France • Germany • India • Japan Luxembourg •
Malaysia • The Netherlands • Russia • Singapore Switzerland

This edition published in the Taylor & Francis e-Library, 2005.

“To purchase your own copy of this or any of Taylor & Francis or Routledge’s collection of thousands of eBooks please go to
www.eBookstore.tandf.co.uk.”

Copyright © 1998 OPA (Overseas Publishers Association) N.V. Published by license under
the Harwood Academic Publishers imprint, part of The Gordon and Breach Publishing
Group.

All rights reserved.

No part of this book may be reproduced or utilized in any form or by any means, electronic
or mechanical, including photocopying and recording, or by any information storage or
retrieval system, without permission in writing from the publisher. Printed in Singapore.

Amsteldijk 166
1st Floor
1079 LH Amsterdam
The Netherlands

British Library Cataloguing in Publication Data

The molecular biology of Alzheimer’s disease

1. Alzheimer’s disease—Molecular aspects

I. Haass, Christian

616.8’31’042

ISBN 0-203-30361-X Master e-book ISBN

ISBN 0-203-34395-6 (Adobe eReader Format)

ISBN: 90-5702-381-4 (Print Edition)

CONTENTS

Contributors	vi
Introduction	x
A Pathology of Alzheimer's Disease	
1. Amyloid Morphology and Pathology of Alzheimer's Disease <i>Haruyasu Yamaguchi</i>	2
2. The Tau Proteins in Alzheimer's Disease <i>Roland Brandt and Jochen Eidenmüller</i>	15
B The Genetics of Familial Alzheimer's Disease	
3. Mutations in Three Genes are Associated with Early Onset Alzheimer's Disease <i>Paul E.Fraser and Peter H.St George-Hyslop</i>	44
C Molecular and Cellular Biology of the β-Amyloid Precursor Protein and Amyloid β-Peptide	
4. The Biological Activities and Function of the Amyloid Precursor Protein of Alzheimer's Disease <i>Gerd Multhaup, Colin L.Masters, Konrad Beyreuther and Roberto Cappai</i>	70
5. Proteolytic Processing of the β -Amyloid Precursor Protein <i>Martin Citron and Dennis J.Selkoe</i>	90
6. Biosynthesis of APP and A β : Multiple Pathways for the Generation of Intracellular A β <i>David G.Cook, Mark S.Forman, Abraham S.C.Chyung, Robert W.Doms and Virginia M.-Y.Lee</i>	105
7. The Cell Biology of Amyloid Precursor Protein <i>Bart De Strooper and Fred Van Leuven</i>	128
8. The Role of Amyloid β -Peptide Terminating at Amino Acid 42 in Early Onset Alzheimer's Disease <i>Steven G.Younkin</i>	147

9. The Biophysics of Amyloid β -Protein Fibrillogenesis 156
David B. Teplow

D The Presenilin Genes

10. The Molecular Biology of Presenilin 1 185
Gopal Thinakaran and Sangram S. Sisodia
11. Molecular Biology of Presenilin 2 199
Wilma Wasco
12. Presenilin Proteins and their Role in Development and Notch Signaling 210
Ralf Baumeister and Christian Haass
13. The Processing of the Amyloid-Precursor-Protein (APP) in Presenilin-1 Deficient Neurons 226
Paul Saftig, Dieter Hartmann, Wim Annaert, Kathleen Craessaerts, Fred Van Leuven and Bart De Strooper
14. Presenilins and their Role in Apoptosis 239
Benjamin Wolozin and James Palacino
15. The Phosphorylation of Presenilin Proteins 269
Jochen Walter and Christian Haass

E Role of Risk Factors in Alzheimer's Disease

16. APOE and its Role in Late Onset Alzheimer's Disease 281
G. William Rebeck

F Transgenic Models for Alzheimer's Disease

17. Transgenic Animal Models in the Development of Therapeutic Strategies for Alzheimer's Disease 298
Matthias Staufenbiel and Bernd Sommer
18. Presenilins in Transgenic Mice 317
Karen Duff

- Index 321

CONTRIBUTORS

Wim Annaert

Experimental Genetics Group
Vlaams Interuniversitair Instituut
voor Biotechnologie (VIB)
Campus Gasthuisberg
K.U.Leuven
B-3000 Leuven
Belgium

Ralf Baumeister

Genzentrum
Ludwig-Maximilians-Universität München
Feodor-Lynen-Strasse 25
81377 München
Germany

Konrad Beyreuther

ZMBH-Center of Molecular Biology
University of Heidelberg
Im Neuenheimer Feld 282
69120 Heidelberg
Germany

Roland Brandt

Department of Neurology
University of Heidelberg
Im Neuenheimer Feld 345
69120 Heidelberg
Germany

Roberto Cappai

Department of Pathology
University of Melbourne and the Mental
Health Research Institute of Victoria
Parkville, Vic 3052
Australia

Martin Citron

Amgen Inc
Department 238, Mailstop 5-1-C
1840 DeHavilland
Thousand Oaks, CA 91320
USA

and

Center for Neurologic Diseases
Brigham and Women's Hospital and
Harvard Medical School
Boston, MA 02115
USA

David G.Cook

Department of Pathology and Laboratory
Medicine
University of Pennsylvania Medical Center
Abramson Research Center
Room 806
34th and Civic Center Boulevard
Philadelphia, PA 19104
USA

Abraham S.C.Chyung

Department of Pathology and Laboratory
Medicine
Third Floor Maloney
HUP
Philadelphia, PA 19104
USA

Kathleen Craessaerts

Experimental Genetics Group
Vlaams Interuniversitair Instituut
voor Biotechnologie (VIB)
Campus Gasthuisberg
K.U.Leuven
B-3000 Leuven
Belgium

Bart De Strooper

Experimental Genetics Group
Vlaams Interuniversitair Instituut
voor Biotechnologie (VIB)
Campus Gasthuisberg
K.U.Leuven
B-3000 Leuven
Belgium

Robert W.Doms

Department of Pathology and Laboratory
Medicine
University of Pennsylvania Medical Center
Abramson Research Center
Room 806
34th and Civic Center Boulevard
Philadelphia, PA 19104
USA

Karen Duff

Mayo Clinic Jacksonville
4500 San Pablo Road
Jacksonville, FL 32224
USA

Jochen Eidenmüller

Department of Neurology
University of Heidelberg
Im Neuenheimer Feld 345
69120 Heidelberg
Germany

Mark S.Forman

Department of Pathology and Laboratory
Medicine
Third Floor Maloney
HUP
Philadelphia, PA 19104
USA

Paul E.Fraser

Centre for Research in Neurodegenerative
Diseases
Departments of Medical Biophysics
and Medicine
University of Toronto
Toronto Hospital
6 Queen's Park Crescent
Toronto, ON M5S 3H2
Canada

Christian Haass

Central Institute of Mental Health

Department of Molecular Biology J5
68159 Mannheim
Germany

Dieter Hartmann

Anatomisches Institut
Universität Kiel
Kiel
Germany

Virginia M.-Y.Lee

Department of Pathology and Laboratory
Medicine
Third Floor Maloney
HUP
Philadelphia, PA 19104
USA

Fred Van Leuven

Experimental Genetics Group
Vlaams Interuniversitair Instituut
voor Biotechnologie (VIB)
Campus Gasthuisberg
K.U.Leuven
B-3000 Leuven
Belgium

Colin L.Masters

Department of Pathology
University of Melbourne and the Mental
Health Research Institute of Victoria
Parkville, Vic 3052
Australia

Gerd Multhaup

ZMBH-Center of Molecular Biology
University of Heidelberg
Im Neuenheimer Feld 282
69120 Heidelberg
Germany

James Palacino

Department of Pharmacology
Bldg. 102, Rm. 3634
Loyola University Medical Center

2160 South First Avenue
Maywood, IL 60513
USA

G. William Rebeck

Alzheimer's Research Center
Massachusetts General Hospital
149 13th Street
Charlestown, MA 02114
USA

Paul Saftig

Zentrum Biochemie und Molekulare
Zellbiologie
Abteilung Biochemie II
Universität Göttingen
Göttingen
Germany

Dennis J. Selkoe

Center for Neurologic Diseases
Brigham and Women's Hospital and
Harvard Medical School
Boston, MA 02115
USA

Sangram S. Sisodia

Department of Pathology and Neuroscience
The Johns Hopkins University School
of Medicine
558 Ross Building
720 Rutland Avenue
Baltimore, MD 21205
USA

Bernd Sommer

Nervous System Research
Novartis Pharma AG
S-356.8.06
4002 Basel
Switzerland

Peter H. St George-Hyslop

Centre for Research in Neurodegenerative
Diseases

Departments of Medical Biophysics
and Medicine

University of Toronto
Toronto Hospital
6 Queen's Park Crescent
Toronto, ON M5S3H2
Canada

Matthias Staufenbiel

Nervous System Research
Novartis Pharma AG
S-356.8.06
4002 Basel
Switzerland

David B. Teplow

Center for Neurologic Diseases
Brigham and Women's Hospital and
Harvard Medical School
Boston, MA 02115
USA

Gopal Thinakaran

Department of Pathology
The Johns Hopkins University School
of Medicine
558 Ross Building
720 Rutland Avenue
Baltimore, MD 21205
USA

Jochen Walter

Central Institute of Mental Health
Department of Molecular Biology J5
68159 Mannheim
Germany

Wilma Wasco

Genetics and Aging Unit
Massachusetts General Hospital East
Building 149, 13th Street
Charlestown, MA 02129-9142
USA

Benjamin Wolozin

Department of Pharmacology
Bldg. 102, Rm. 3634
Loyola University Medical Center
2160 South First Avenue
May wood, IL 60513
USA

Haruyasu Yamaguchi
Gunma University School of Health Sciences
3-39-15 Showa-Machi
Maebashi, Gunma 371
Japan

Steven G. Younkin
Mayo Clinic Jacksonville
4500 San Pablo Road
Jacksonville, FL 32224
USA

INTRODUCTION

Few topics in modern biomedical research have generated as much public attention as the discovery of molecular and genetic mechanisms underlying Alzheimer's disease (AD). The profound public awareness is due to the fact that the number of AD cases is dramatically increasing in all civilized countries. Whereas Alois Alzheimer in his lifetime found only two cases of this devastating disease, today almost every reader of this book knows someone affected by AD. The average life expectancy in the last century was approximately 37 years. This has changed dramatically in the 20th century, and today the mean life expectancy exceeds 73 years. This demographic fact guarantees that AD will generate one of the most important health problems world wide. This has not been fully recognized by many politicians. The tremendous increase of AD patients at the turn of the century will have severe implications for our social security—and health insurance systems.

With the increasing public awareness about AD more funding has become available and considerable research efforts have gone into the search for the genes and molecular mechanisms responsible for AD. World wide, scientists of academic institutions and the pharmaceutical industry put a lot of effort and money into the discovery of genes and molecular mechanisms involved in AD. During the last 10 years our knowledge about the mechanisms causing AD has increased substantially. There is almost no other field in modern biomedical research which has made such pronounced progress as our understanding of the pathological mechanisms causing AD. Within a very short time three genes were identified which are clearly involved in the genetically inherited forms of the disease (familial Alzheimer's disease, FAD). Moreover, simple tissue culture systems are now available to analyze the molecular effects of mutations in these genes on amyloid β -peptide ($A\beta$) generation. The same culture system is also used to identify drugs which may be capable of inhibiting production of the neurotoxic $A\beta$ peptide. During the last two years it became clear that a longer form of $A\beta$ (containing 42 instead of 40 amino acids) appears to be the major culprit responsible for the disease. Generation of the 42 amino acid $A\beta$ ($A\beta$) is now known to be increased by FAD associated mutations found in three different genes (Figure 1). Moreover, the accumulation of intracellular $A\beta_{42}$ leads to the possibility that the toxic effects of this highly amyloidogenic peptide are exerted prior to its secretion independent of amyloid plaque formation (Figure 1). Such a putative mechanism might also solve the apparent discrepancy that $A\beta_{42}$ levels determined in biological fluids do not reach the critical concentration

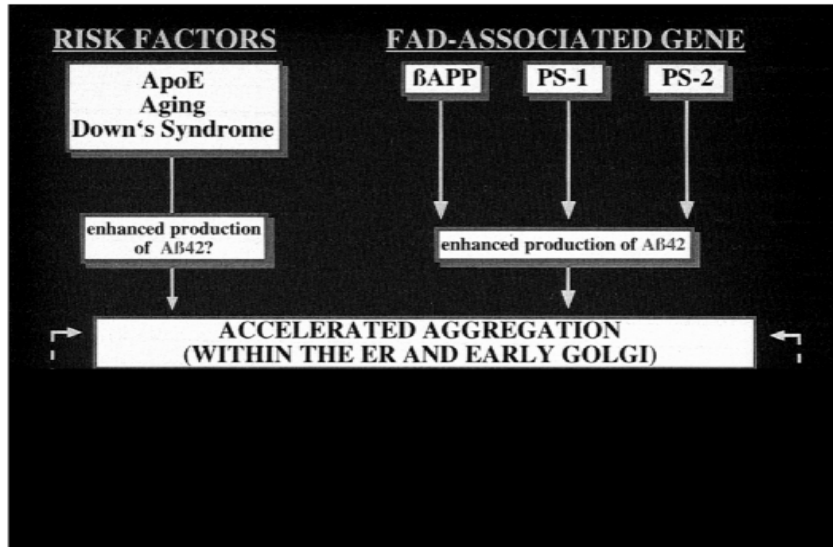


Figure 1.

required for aggregation since intracellular accumulation of A β 42 could very well lead to high local concentrations of A β 42 sufficient for its precipitation.

In addition to the autosomal dominant mutations, a polymorphism in the ApoE gene has been demonstrated to be responsible for numerous late onset cases of AD. Brains of patients homozygous for the ApoE4 allele show an increased amyloid plaque density, thus again supporting a central role for A β aggregation in AD pathology (Figure 1).

In parallel to our knowledge of the cellular and molecular events causing AD, we are also making great progress in the understanding of A β fibrillogenesis. This field will be of importance for the understanding of amyloid plaque formation and the generation of drugs inhibiting A β aggregation.

Transgenic mice models, reproducing several aspects of human AD pathology are now available in several laboratories. These mice will greatly facilitate basic research on the progression of AD pathology in brain tissue, but are also valuable reagents for *in vivo* drug testing. Moreover, very recently novel animal models were introduced into the field. Research on the nematode *Caenorhabditis elegans* allowed the very rapid discovery of a biological function of two FAD causing genes. This system is now available to help identify further genetic factors playing a major role in AD pathology.

While the findings above described clearly support a central role of A β 42 in the amyloid cascade hypothesis (Figure 1), additional pathological factors such as tangle formation and inflammatory processes are certainly essential for the initiation of neuronal cell death. Developments in these areas and their interplay in the total pathophysiology of AD continues to be vital for our understanding of pathological mechanisms and future treatment or prevention of AD.

In this book some of the most distinguished AD researchers will describe their recent findings. It is my great hope that this book will provide an introduction into the latest and the most timely aspects of AD research and will enable scientists in related research fields as well as physicians working with AD patients to obtain a quick and complete overview of the current state of the art in one of the most exciting fields in neuroscience research.

I would like to thank the authors for their contributions to this book. Due to the rapid and coordinated work of all authors it is possible to publish a comprehensive summary of our current knowledge of the molecular mechanism involved in AD pathobiology.

Christian Haass

PATHOLOGY OF ALZHEIMER'S DISEASE

1. AMYLOID MORPHOLOGY AND PATHOLOGY OF ALZHEIMER'S DISEASE

HARUYASU YAMAGUCHI

*Gunma University School of Health Sciences, 3-39-15 Showa-machi, Maebashi 371,
Japan*

INTRODUCTION

Amyloid is an extracellular mass of amyloid fibrils. The fibrils are 7–10 nm in diameter under electron microscopy (EM). In the brain, amyloids consist of amyloid β protein ($A\beta$), and they are associated with proteins such as α 1-antichymotrypsin, apolipoprotein E, apolipoprotein J, vitronectin and non- $A\beta$ component (NAC). Of these proteins, only NAC can be biochemically extracted from the deposited amyloid (Ueda *et al.*, 1993). In the immunohistochemical study, labeling of senile plaques is different among antisera against these associated proteins. For example, anti-apolipoprotein E labels most plaques (Figure 1a) (Yamaguchi *et al.*, 1994). Anti-apolipoprotein J labels neurons, amyloid angiopathy and a subset of senile plaques (Figure 1b).

There are many forms of cerebral amyloid deposits, and the forms depend on the sites of deposition: senile plaques in the neuropil, amyloid plaques in the white matter, subpial β amyloid deposits, β amyloid deposits on extracellular neurofibrillary tangles (E-NFT), small stellate deposits, intra-glial $A\beta$ -positive granules, and amyloid angiopathy (Table 1). Senile plaques are further subdivided into diffuse, primitive, typical and compact (burnedout) plaques. First, the author describes the morphological characteristics of cerebral β amyloid deposition, and then, explains the significance of cerebral β amyloid deposition in the pathology of Alzheimer's disease (AD).

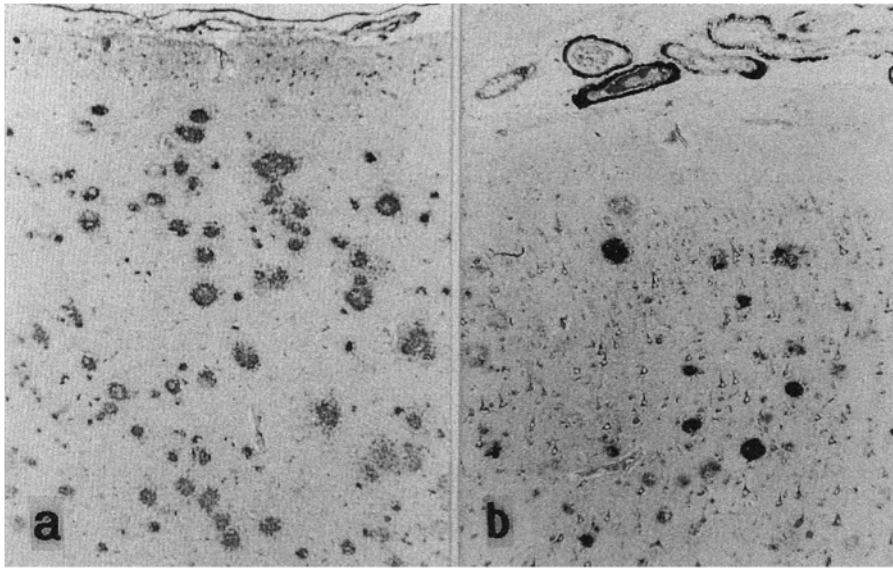


Figure 1 (a) Anti-apolipoprotein E labels most senile plaques, (b) Anti-apolipoprotein J labels neuron, amyloid angiopathy and a subset of senile plaques.

Table 1. Forms of A β deposits and their components.

<i>Forms of the Aβ deposits</i>	<i>Aβ</i>		<i>Neuritic reaction</i>	<i>Glial reaction</i>
	<i>Amounts</i>	<i>Fibril</i>		
Diffuse plaques				
Early stage	+	Non-fibrillar	–	–
Advanced stage	++	Fibrillar	–/+	+
Primitive plaques	+++	Fibrillar	++	++
Typical plaques	++++	Fibrillar	+++	++
Compact plaques	+++	Fibrillar	–	+
Small stellate deposits	+	non-fib./Fib.	–	–/+?
Astroglial A β granules	+	Non-fibrillar (Intra-cellular deposits)		
Subpial deposits	+	Non-fib./Fib.	–	+?
A β deposits on E-NFT	+	Fibrillar	++	+++
Amyloid angiopathy	++++	Fibrillar		

MORPHOLOGY

Characteristics of the each form of cerebral β amyloid deposits are summarized in [Table 1](#).

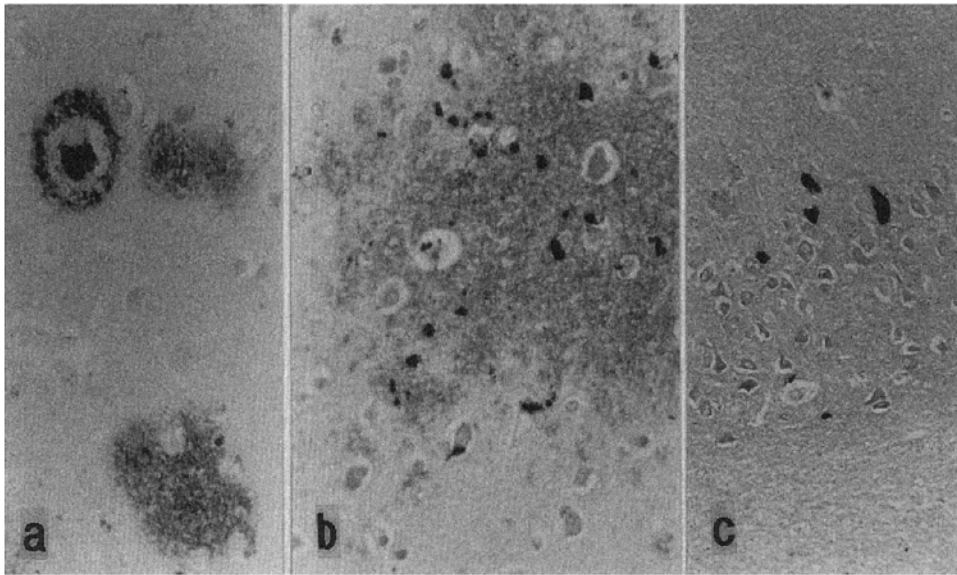


Figure 2 (a) High power view of the typical (upper left) and diffuse plaques, (b) Diffuse plaques associated with clusters of intensely A β -positive granules in astrocytes. (c) A subset of extracellular neurofibrillary tangles is A β -positive in CA1. A β labeling.

Senile Plaques

The site of plaque formation is an important factor that determines plaque type. This means that some areas have their own plaque types. For example, the diffuse type is predominant in the molecular layer of the cerebellum, and the typical type is predominant in the Purkinje cell layer. In the cerebral cortex, motor and visual cortices tend to show predominantly typical plaques, while association cortices tend to show abundant diffuse/primitive plaques. Senile plaques are subdivided into following 6 types, although they overlap (Yamaguchi *et al.*, 1988).

Typical plaques

Typical (or classical) plaques (Figure 2a) consist of 3 major components; amyloid (central core and crown), swollen neurites, and reactive glia. The central core is a packed mass of amyloid fibrils, and that is surrounded by astroglial and microglial processes. Amyloids in the crown area are scattered bundles of amyloid fibrils, which are usually surrounded by glial processes. Swollen neurites contain dense bodies, and can be labeled consistently with ubiquitin antibodies. They contain paired helical filaments (PHF) in AD brains, but usually do not in control (non-demented) brains (Probst *et al.*, 1989). Precise EM study of typical plaques has shown a close association of the central cores with capillaries (Miyakawa *et al.*, 1986). This type tends to appear with amyloid angiopathy. These findings suggest a vascular origin of typical plaques. I think that plaque evolution from primitive to typical plaques is not a common course. The typical plaque may develop from the small central core (i.e.

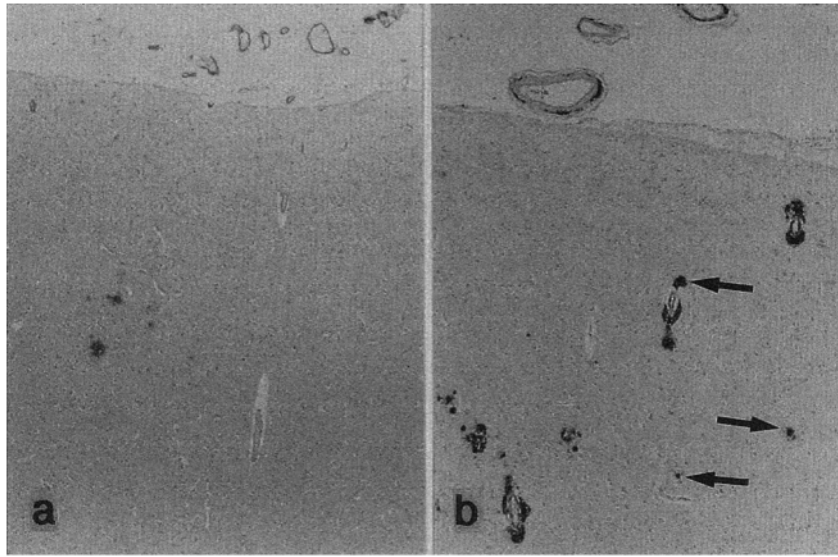


Figure 3 (a) Only this area has diffuse plaques in the frontal cortex of 49 year old subject, (b) Amyloid angiopathy is predominant in this 59 year-old subject. Small cores (arrows) may be in an early stage of typical plaques. A β labeling.

compact plaques) (Fischer, 1910). Typical plaques tend to appear in the deep neocortical layers, while diffuse plaques and small stellate deposits are common in the superficial neocortical layers (lamina-specific pathology; Delaere *et al.*, 1991). This finding also suggest that typical plaques do not develop from diffuse plaques.

Primitive plaques

Primitive plaques consist of the same 3 components as typical plaques, although the amounts of amyloid are smaller. Amyloids exist as scattered bundles of fibrils like the crown area of typical plaques. Scattered amyloids are surrounded by astroglial and microglial processes. Swollen neurites in primitive plaques are less prominent than in typical plaques.

Diffuse plaques

The contents of diffuse plaques (Figures 2a and 3a) are not consistent. In the early stage of diffuse plaques, they contain amorphous extracellular A β deposits instead of amyloid fibrils (Yamaguchi *et al.*, 1989a, 1990, 1991a). These early diffuse plaques are also called pre-plaques or pre-amyloid deposits. They lack glial reaction and swollen neurites. In the advanced stage of diffuse plaques, small amounts of amyloid fibrils are found between cell processes, some of which are astroglial in origin (Figure 4). The glial reaction is weak, and some plaques have small swollen neurites that can be seen under EM (Yamaguchi *et al.*, 1991a). Normal neurons are frequently seen within diffuse plaques. In the AD cerebral cortex, some diffuse plaques are seen in the early stages, but more are in the advanced

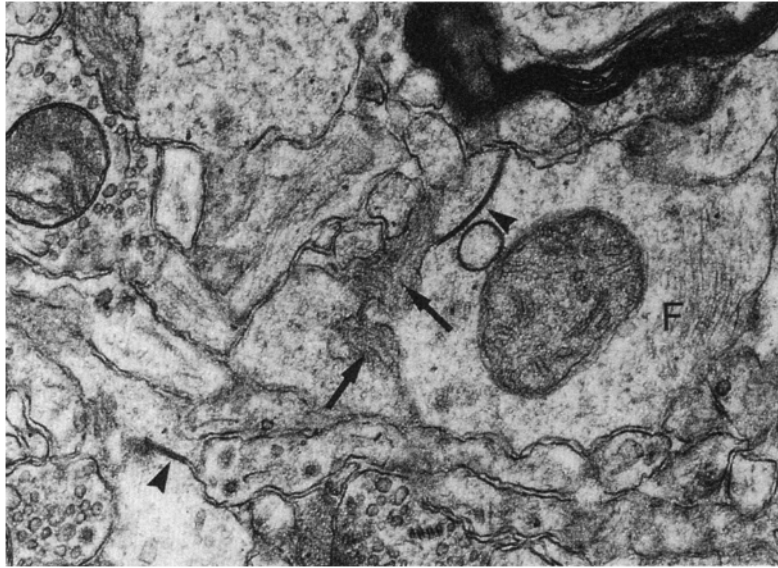


Figure 4 Ultrastructure of diffuse plaques. In the advanced stage, amyloid fibrils (arrows) are seen between astroglial processes, which have gap junction (arrowheads) and glial filaments (F).

stages. Therefore, neocortical diffuse plaques in AD are not necessarily newly formed. Diffuse plaques that appear in the brains of teenager Down's subjects or in the brains of middle-aged non-demented subjects may be in the early stages. These subjects are useful to learn about the early stages of plaque formation.

Diffuse plaques associated with clusters of intra-astroglial A β -positive granules

This novel type of A β deposition was recently discovered (Yamaguchi *et al.*, 1998). A subset of diffuse plaques was associated with clusters of intensely A β -positive granules (Figure 2b). These granules appeared very close to the astroglial nucleus, and were localized to lipofuscin granules of astrocytes by dual immunolabeling of A β and astroglial marker antibodies. The plaque part of the novel diffuse plaques associated with astroglial A β is very weakly A β -positive. The N-terminal of A β in astroglial A β granules is truncated and shorter than that in plaque part, indicating degradation of A β in the astroglial cytoplasm (lysosome?). Intra-cellular A β granules may represent incorporation of deposited A β , and diffuse plaques associated with astroglial A β granules may be in the disappearing stage. This type is found in about one third of plaque-bearing brains from mentally normal subjects. Akiyama *et al.* (1996) also reported similar glial A β , although they found A β granules in the microglia rather than in astrocytes in the AD brains. It is important to distinguish astroglial A β granules from small stellate deposits. Intra-glial A β -positive granules (1–2 μ m in diameter) form clusters (5–10 μ m in diameter), and are usually seen adjacent to the astroglial nucleus. Small stellate deposits (Figure 5b) are stellate in shape. They are extracellular, and therefore, quite different from astroglial A β granules.

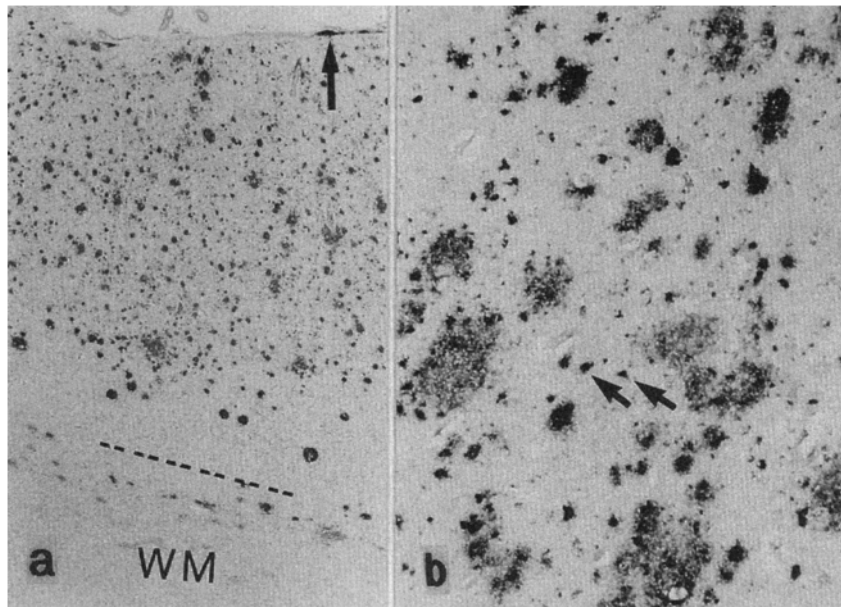


Figure 5 (a) In the Alzheimer brain, amyloid deposits are seen in the subcortical white matter (WM). Dot line indicates the junction between gray and white matter. Arrow indicates subpial A β deposits, (b) Small stellate deposits are seen among diffuse and primitive plaques in the Alzheimer brain. A β labeling.

Compact (burned-out) plaques

A compact (burned-out) plaque is a mass of packed amyloid fibrils, which is similar to the central core of typical plaques (Figure 3b). The name “burned-out” indicates that this type shows the disappearing stages of typical plaques. However, burned-out plaques appear even in the neocortex of non-demented middle-aged subjects (Figure 3b). In our study of control subjects aged between 40 and 59 years old (Sugihara *et al.*, 1995), about two thirds of the plaque-bearing subjects predominantly showed diffuse plaques (Figure 3a), whereas about one third predominantly showed typical and compact plaques (Figure 3b). The latter subjects were usually associated with amyloid angiopathy. From this finding, I think that some “burned-out” plaque may be an early stage of the typical plaque as suggested by Fischer in 1910. The small amyloid core may form around the capillary, and then the core becomes bigger. Then, this “burned-out” plaque may become associated with surrounding amyloid deposits (crown area) and neuritic changes, and finally becomes a typical plaque. The term “compact plaques” is more appropriate than “burned-out plaques”.

Amyloid plaques in the white matter

Together with numerous plaque formation in the gray matter, single or clusters of small amyloid masses are seen in the neocortical white matter of AD subjects (Figure 5a). This type of plaque is rarely seen in the mentally normal subjects. The amyloid plaques in the white matter represent massive A β deposits in the area.

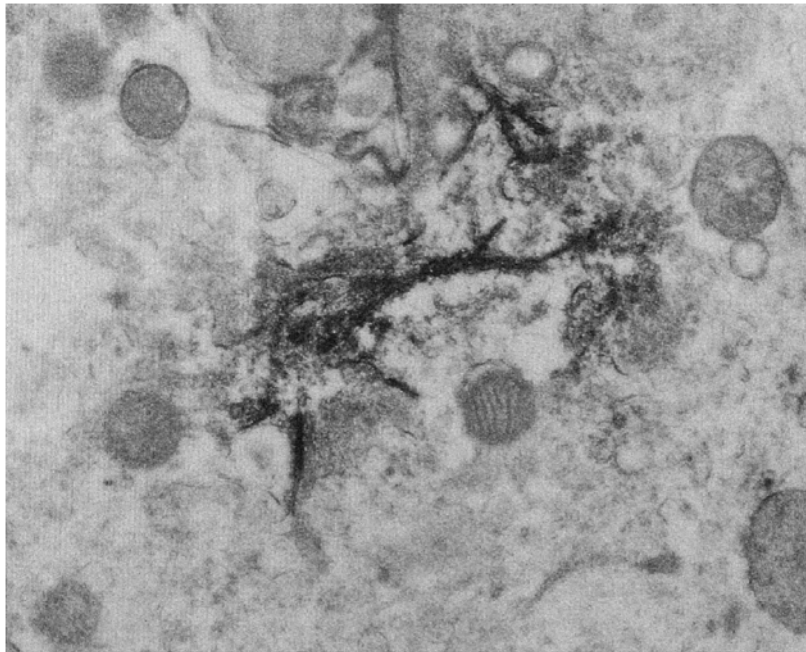


Figure 6 Immunoelectron micrograph of small A β deposits. A β deposits appear between plasma membranes of cell processes. A β labeling.

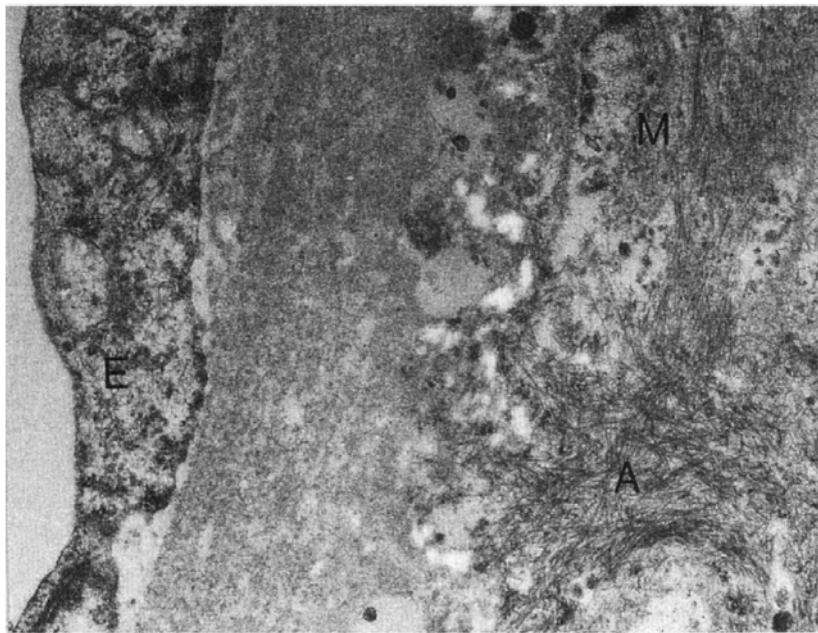


Figure 7 Electron micrograph of amyloid angiopathy in a meningeal small artery. Amyloid fibrils (A) appear in the basement membrane between smooth muscle cells (M). Letter E indicates endothel.

Subpial β Amyloid Deposits

Subpial β amyloid deposition is found in both AD and mentally-normal brains (Figure 5a). The subpial areas consist of thick processes of astrocytes forming glia limitans. Amyloid fibrils appear as small bundles between astroglial processes, but sometimes it is difficult to detect amyloid fibrils in the A β -positive area (Yamaguchi *et al.*, 1991b). A β may exist in both fibrillar and non-fibrillar forms (Table 1).

β Amyloid Deposits on Extracellular-NFT

Extracellular NFT (E-NFT) is one of the preferential sites of cerebral β amyloid deposition (Figure 2c) (Yamaguchi *et al.*, 1991c). Some, but not all, of E-NFT becomes A β -positive, but it occurs only when senile plaques appear in the vicinity. Therefore, A β deposits are a secondary event, which occurs in a subset of E-NFT. E-NFT consist of remnant PHF bundles, and astroglial and microglial processes, and are frequently associated with swollen neurites like senile plaques (Yamaguchi *et al.*, 1991d). Bundles of amyloid fibrils were detected in A β -positive E-NFT. The entorhinal cortex, CA1 and subiculum are common sites of this lesion. A β -positive E-NFT are common in AD, but rare in mentally normal subjects.

Small Stellate Deposits

In the AD neocortex, numerous small stellate A β -immunoreactive deposits are found between senile plaques (Figure 5b). They are also found in mentally-normal subjects with considerable amounts of senile plaques. In an immuno-EM study, this small A β -positive area contained small bundles of amyloid fibrils or an amorphous substance between cell processes, and the plasma membrane of the adjacent processes were also A β -positive (Figure 6) (Yamaguchi *et al.*, 1990). These lesions do not seem to grow larger to become a diffuse/primitive plaque. Most of the small stellate deposits continue to be small in size.

Amyloid Angiopathy

Amyloid deposition in the vessel wall is found in both AD and mentally-normal subjects. Preferential sites of amyloid angiopathy are meningeal arteries in the subarachnoid space and penetrating arteries. In the small arterial wall, amyloids start depositing in the basement membrane as small bundles of fibrils (Figure 7) (Yamaguchi *et al.*, 1992). Then, amounts of amyloid fibrils increase, and smooth muscle cells die and are replaced by amyloids. In the large meningeal artery, amyloids first appear at the junction between the media and adventitia. In the capillary wall, amyloids also appear in the basement membrane, and then spread outside into the neuropil.

A β Deposits in the Extra-neural Tissues

A β immunolabeling of subcutaneous vessels in skin biopsy material was reported to have a diagnostic significance for AD (Joachim *et al.*, 1989). Recently, A β -immunoreactivity was reported in the

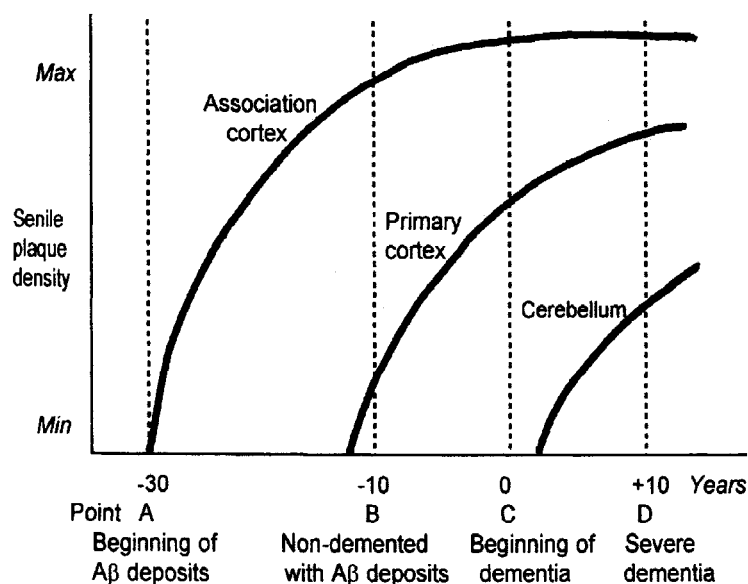


Figure 8 Progression of the senile plaque formation in a senile case of Alzheimer's disease. Cerebral A β deposits begins 30 years prior to the beginning of dementia (Points A and C). If the patient died at Point B, pathological diagnosis is "A β deposits within a range of normal aging".

rimmed vacuoles (lysosome origin) of degenerating human skeletal muscles (Leclerc *et al.*, 1993), and chloroquine-induced myopathy in rat soleus muscles (Tsuzuki *et al.*, 1995). In the normal human kidney, A β -immunoreactivity was found in the renal tubules with transthyretin immunoreactivity (Tsuzuki *et al.*, 1997). However, amyloid fibrils were not found under EM in these extra-neural A β -positive lesions.

SIGNIFICANCE OF THE β AMYLOID DEPOSITS IN THE ALZHEIMER PATHOLOGY

A β Deposits in Disorders Other Than Alzheimer's Disease

Although A β deposits are found in some disorders other than AD, most are related to the pathogenesis of AD: 1.5 times over dose of amyloid β protein precursor (APP) gene in Down's syndrome; point mutation of APP gene at the β protein portion in hereditary cerebral hemorrhage with amyloidosis-Dutch type (Levy *et al.*, 1990); high apolipoprotein E ϵ 4 allele frequency in diffuse Lewy body disease (St. Clair *et al.*, 1994); and possible overexertion of APP gene in dementia pugilistica (Roberts *et al.*, 1990). Massive A β deposits in the neocortex is highly specific to AD, whereas tau pathology (NFT formation) occurs in a variety of diseases.

β Amyloid Deposits in the Mentally Normal Subjects and Pathological Diagnosis of the Alzheimer's Disease

The presence of numerous senile plaques in neocortex is an essential finding for the pathological diagnosis of AD. No AD brains lack neocortical senile plaque formation. However, A β deposits occur in the process of brain aging, and many elderly subjects have considerable amounts of cerebral A β deposits without showing dementia (Figure 8, Point B). We have shown in a study on 126 non-demented subjects who died with malignant neoplasms that cerebral A β deposits are first found in the fifth decade (Figure 3a), and its prevalence gradually increases with age (Sugihara *et al.*, 1995). Together with the fact that the prevalence of AD increases in the eighth decade, the author suggests that it takes 30 years from the start of neocortical A β deposits (Figure 8, Point A) to clinical manifestation of dementia (Point C). The time lag was first estimated in the Down's syndrome subjects, where A β deposition starts in the third decade, and mental decline begins in the sixth decade, suggesting the time lag of 30 years (Mann *et al.*, 1989). During the time lag, density (number/area) of the senile plaques in the association cortex gradually increases and reaches a plateau (Point B) before the beginning of dementia (Point C). Mann *et al.*, (1988) compared the plaque density between biopsied and autopsied samples from the same AD patient, and showed that in the association cortex, plaque density did not greatly increase after the beginning of dementia. The similar plaque density among Points A, B and C of Figure 8 makes it difficult to diagnose AD by the plaque density of the neocortical (i.e. frontal) tissue sections. During the time lag of 30 years, sites of senile plaque formation, however, continue to increase from the association cortex to primary cortical areas, such as the visual and motor cortices (Braak *et al.*, 1997). When the subject show severe dementia (Point D), senile plaques were frequently found in cerebellum and brainstem, and less frequently spinal cord. This means that the distribution pattern (involved area) of the senile plaques is quite important for the diagnosis of AD. As summarized in Table 2, senile plaques are common in the motor and visual cortices of the AD subjects (Point D), whereas rare in those of the mentally-normal subjects (Point B). In the cerebellum, senile plaque formation is common in the AD subjects (Point D), whereas it is quite rare in the non-demented subjects (Yamaguchi *et al.*, 1989b). Senile plaque formation in brainstem and spinal cord do not occur in the process of normal aging (Ogomiri *et al.*, 1989). A β deposits in the subcortical white matter is also rare in the non-demented subjects. In summary, exact distribution (involved areas), but not exact density (number/area), of the senile plaques should be evaluated for the histopathological diagnosis of AD (Table 2). As shown in Figure 8, non-demented elderly subjects gradually develop AD pathology, and therefore the AD in elderly subjects is not a discrete disease entity, but a terminal of the brain aging.

In the neocortex, accumulation of abnormally phosphorylated tau as a form of NFT, neuropil threads and plaque associated neurites follow the A β deposits, and this reaction is much more intense in AD (Point D) than in non-demented subjects (Point B). Degree of tau pathology is also an important findings for the pathological diagnosis, if present together with A β deposits.

Plaque Density and Clearance of the Plaques

As described in the “Diffuse plaques associated with clusters of intra-astroglial A β -positive granules” session, we found “disappearing” senile plaques in the neocortex of non-demented subjects (Figure 2b) (Yamaguchi *et al.*, 1998). Astrocytes and microglia can remove plaque amyloid. In rat brain, injected plaque cores were soon removed by phagocytic cells (Frautschy *et al.*, 1992). In the pancreas of transgenic mice over-expressing A β gene, macrophages incorporate β amyloid (Kawarabayashi *et al.*, 1996). In general, amyloid

Table 2. Difference of senile palque distribution between Alzheimer’s Disease and non-demented subjects with considerable amounts of cerebral A β deposits.

<i>Sites</i>	<i>Alzheimer’s disease</i>	<i>Non-demented with Aβ deposits</i>
Association area		
Gray	++	++
Subcortical white	+	—
Primary area	+	-/+
Cerebellum	+ or -/+	—
Brain stem	-/+	—
Spinal cord	-/+	—

++: high density; +moderate density;

-/+ : low density if present; and—: quite rare

fibrils can be gradually removed, if production of amyloid stopped. This character is quite different from paired helical filaments of NFT, which are resistant to phagocytosis and remain for long time after the neuronal cell death. Our findings suggest that senile plaques can be removed in the early stage of plaque formation. A dynamic balance between activities of plaque formation and plaque destruction determines senile plaque density. This hypothesis suggests the possibility to develop effective treatment. Between the points A and B of Figure 8, promotion of plaque clearance activity may delay the beginning of dementia. Further research on the mechanism of plaque destruction is as important as that of plaque production.

REFERENCES

- Akiyama, H., Schwab, C., Kondo, H., Mori, H., Kametani, F., Ikeda, K., and McGeer, P.L. (1996) Granules in glial cells of patients with Alzheimer’s disease are immunopositive for C-terminal sequences of β -amyloid protein. *Neurosci. Lett.*, **206**, 169–172.
- Braak, H., and Braak, E. (1997) Pattern of cortical lesions in Alzheimer’s disease. In K.Iqbal, B. Winblad , T.Nishimura, M.Takeda, and H.M.Wisniewski, (eds.), *Alzheimer’s Disease: Biology, Diagnosis and Therapeutics*, Wiley, England, pp. 227–237.

- Delaere, P., Duyckaerts, C., He, Y., Piette, F., and Hauw, J.J. (1991) Subtypes and differential laminar distributions of β A4 deposits in Alzheimer's disease: relationship with the intellectual status of 26 cases. *Acta Neuropathol.*, **81**, 328–335.
- Fischer, O. (1910) Die presbyophrone Demenz, deren anatomische Grundlage und klinische Abgrenzung. *Z. Gesamte Neurol Psychiatr.*, **3**, 371–471.
- Frautschy, S.A., Cole, G.M., and Baird, A. (1992) Phagocytosis and deposition of vascular β -amyloid in rat brains injected with Alzheimer β -amyloid. *Am. J. Pathol.*, **140**, 1389–1399.
- Joachim, C.L., Mori, H., and Selkoe, D.J. (1989) Amyloid β -protein deposition in tissues other than brain in Alzheimer's disease. *Nature*, **341**, 226–320.
- Kawarabayashi, T., Shoji, M., Sato, M., Sasaki, A., Ho, L., Eckman, C.B., Prada, C.M., Younkin, S.G., Kobayashi, T., Tada, N., Matsubara, E., Iizuka, T., Harigaya, Y., Kasai, K., and Hirai, S. (1996) Accumulation of β -amyloid fibrils in pancreas of transgenic mice. *Neurobiol. Aging*, **17**, 215–222.
- Leclerc, A., Tome, F.M., and Fardeau, M. (1993) Ubiquitin and beta-amyloid-protein in inclusion body myositis (IBM), familial IBM-like disorder and oculopharyngeal muscular dystrophy: an immunocytochemical study. *Neuromuscul. Disord.*, **3**, 283–291.
- Levy, E., Carman, M.D., Fernandez Gonzalez, J., Power, M.D., Lieberburg, I., van Duinen, S.G., Bots, G.T., Luyendijk, W., Shelanski, M.L., and Frangione, B. (1990) Mutation of Alzheimer's disease amyloid gene in hereditary cerebral hemorrhage, Dutch type. *Science*, **248**, 1124–1126.
- Mann, D.M.A., Marcyniuk, B., Yates, P.O., Neary, D., and Snowden, S. (1988) The progression of the pathological changes of Alzheimer's disease in frontal and temporal neocortex examined both at biopsy and at autopsy. *Neuropathol Appl. Neurobiol.*, **14**, 177–195.
- Mann, D.M.A., and Esiri, M.M. (1989) The pattern of acquisition of plaques and tangles in the brains of the patients under 50 years of age with Down's syndrome. *J. Neurol Sci.*, **89**, 169–179.
- Miyakawa, T., Katsuragi, S., Watanabe, K., Shimoji, A., and Ikeuchi, Y. (1986) Ultrastructural studies of amyloid fibrils and senile plaques in human brain. *Acta Neuropathol.*, **70**, 202–208.
- Ogomori, K., Kitamoto, T., Tateishi, J., Sato, Y., Suetsugu, M., and Abe, M. (1989) Beta-protein amyloid is widely distributed in the central nervous system of patients with Alzheimer's disease. *Am. J. Pathol.*, **134**, 243–251.
- Probst, A., Anderton, B.H., Brion, J.P., and Ulrich, J. (1989) Senile plaque neurites fail to demonstrate anti-paired helical filament and anti-microtubule-associated protein-tau immunoreactive proteins in the absence of neurofibrillary tangles in the neocortex. *Acta Neuropathol.*, **77**, 430–436.
- Roberts, G.W., Allsop, D., and Bruton, C. (1990) The occult aftermath of boxing. *J. Neurol. Neurosurg. Psychiatr.*, **53**, 373–378.
- St. Clair, D., Norrman, J., Perry, R., Yates, C., Wilcock, G., and Brookes, A. (1994) Apolipoprotein E ϵ 4 allele frequency in patients with Lewy body dementia, Alzheimer's disease and age-matched controls. *Neurosci. Lett.*, **176**, 45–46.
- Sugihara, S., Ogawa, A., Nakazato, Y., and Yamaguchi, H. (1995) Cerebral β amyloid deposition in patients with malignant neoplasms: its prevalence with aging and effects of radiation therapy on vascular amyloid. *Acta Neuropathol.*, **90**, 135–141.
- Tsuzuki, K., Fukatsu, R., Takamaru, Y., Yoshida, T., Hayashi, Y., Yamaguchi, H., Fujii, N., and Takahata, N. (1995) Amyloid β protein in rat soleus muscle in chloroquine-induced myopathy using end-specific antibodies for A β 40 and A β 42: immunohistochemical evidence for amyloid β protein. *Neurosci. Lett.*, **202**, 77–80.

- Tsuzuki, K., Fukatsu, R., Hayashi, Y., Yoshida, T., Sasaki, N., Takamaru, Y., Yamaguchi, H., Tateno, M., Fujii, N., and Takahata, N. (1997) Amyloid β protein and transthyretin, sequestering protein colocalize in normal human kidney. *Neurosci. Lett.*, **222**, 163–166.
- Ueda, K., Fukushima, H., Masliah, e., Xia, Y., Iwai, A., Yoshimoto, M., Otero, D.A.C., 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.
- Yamaguchi, H., Hirai, S., Morimatsu, M., Shoji, M., and Ihara, Y. (1988) A variety of cerebral amyloid deposits in the brains of Alzheimer-type dementia demonstrated by β protein immunostaining. *Acta Neuropathol.*, **76**, 541–549.
- Yamaguchi, H., Nakazato, Y., Hirai, S., Shoji, M., and Harigaya, Y. (1989a) Electron micrograph of diffuse plaques: initial stage of senile plaque formation in the Alzheimer brain. *Am. J. Pathol.*, **135**, 593–597.
- Yamaguchi, H., Hirai, S., Morimatsu, M., Shoji, M., and Nakazato, Y. (1989b) Diffuse type of senile plaques in the cerebellum of Alzheimer-type dementia demonstrated by β protein immunostain. *Acta Neuropathol.*, **77**, 314–319.
- Yamaguchi, H., Nakazato, Y., Hirai, S., and Shoji, M. (1990) Immunoelectron microscopic localization of amyloid β protein in the diffuse plaques of Alzheimer-type dementia. *Brain Res.*, **508**, 320–324.
- Yamaguchi, H., Nakazato, Y., Shoji, M., Takatama, M., and Hirai, S. (1991a) infrastructure of diffuse plaques in senile dementia of the Alzheimer type: comparison with primitive plaques. *Acta Neuropathol.*, **82**, 13–20.
- Yamaguchi, H., Nakazato, Y., Yamazaki, T., Shoji, M., Kawarabayashi, T., and Hirai, S. (1991b) Subpial β /A4 amyloid deposition occurs between astroglial processes in Alzheimer-type dementia. *Neurosci. Lett.*, **123**, 217–220.
- Yamaguchi, H., Nakazato, Y., Shoji, M., Okamoto, K., Ihara, Y., Morimatsu, M., and Hirai, S. (1991c) Secondary deposition of β amyloid within extracellular neurofibrillary tangles in Alzheimer-type dementia. *Am. J. Pathol.*, **138**, 699–705.
- Yamaguchi, H., Nakazato, Y., Kawarabayashi, T., Ishiguro, K., Ihara, Y., Morimatsu, M., and Hirai, S. (1991d) Extracellular neurofibrillary tangles associated with degenerating neurites and neuropil threads in Alzheimer-type dementia. *Acta Neuropathol.*, **81**, 603–609.
- Yamaguchi, H., Yamazaki, T., Lemere, C.L., Frosch, M.P., and Selkoe, D.J. (1992) Beta amyloid is focally deposited within the outer basement membrane in the amyloid angiopathy of Alzheimer's disease: an immunoelectron microscopic study. *Am. J. Pathol.*, **141**, 249–259.
- Yamaguchi, H., Ishiguro, K., Sugihara S., Nakazato Y., Kawarabayashi T., Sun X., and Hirai S. (1994) Presence of apolipoprotein E on extracellular neurofibrillary tangles and on meningeal blood vessels precedes the Alzheimer β amyloid deposition. *Acta Neuropathol.*, **88**, 413–419.
- Yamaguchi, H., Sugihara, S., Saido, T.C., Ihara, Y., and Nakazato, Y. (1998) Diffuse plaques associated with astroglial amyloid β protein, possibly showing a disappearing stage of senile plaques. *Acta Neuropathol.*, **95**, 217–222.

2.

THE TAU PROTEINS IN ALZHEIMER'S DISEASE

ROLAND BRANDT* AND JOCHEN EIDENMÜLLER

*Department of Neurobiology, University of Heidelberg, Im Neuenheimer Feld 345,
D-69120 Heidelberg, Germany*

INTRODUCTION

Alzheimer's disease (AD) is characterized by two histopathological hallmarks—amyloid plaques and neurofibrillary tangles (NFTs). Whereas plaque formation can occur without dramatic loss of neurons, tangle formation closely correlates with neuronal cell death both temporally and spatially during the progression of disease.

In the 80s, tau—a neuronal microtubule-associated protein (MAP), which normally is enriched in axons *in situ*—has been identified as the major component of NFTs. The involvement of a cytoskeletal component in AD and the presence of a potentially direct link between tangle formation and neurodegeneration prompted many studies on the role of tau in AD. However, despite an ever increasing body of data on the many changes which tau undergoes in disease, its role regarding fundamental disease processes is still unclear. For example, direct evidence for a primary role of tau modification in tangle formation or cell death is still missing. Moreover, the function of tau during normal brain development remains unsolved, which complicates an understanding of tau in a disease that develops over a time scale of decades and may involve sudden changes in the behavior of a variety of proteins and other factors.

Evaluating tau's role in disease process first requires a proper understanding of tau's role during normal brain development and maintenance of neurons. This is approached in the first part of this chapter, where studies on the role of tau in normal brain function are reviewed. In the second part, data on the structural and functional changes that tau undergoes during AD are summarized. In the last part, experimental models to understand the role of tau in AD pathology are described and, finally, a scheme to conceptualize the available data is presented. Naturally, this part is the most hypothetical section of this review and directly links to many open questions which require experimental testing.

TAU AND BRAIN FUNCTION

A family of Closely Related Phosphoproteins

Tau was originally identified as the low-molecular weight component of a protein fraction that copurifies with brain microtubules during repeated assembly/disassembly cycles

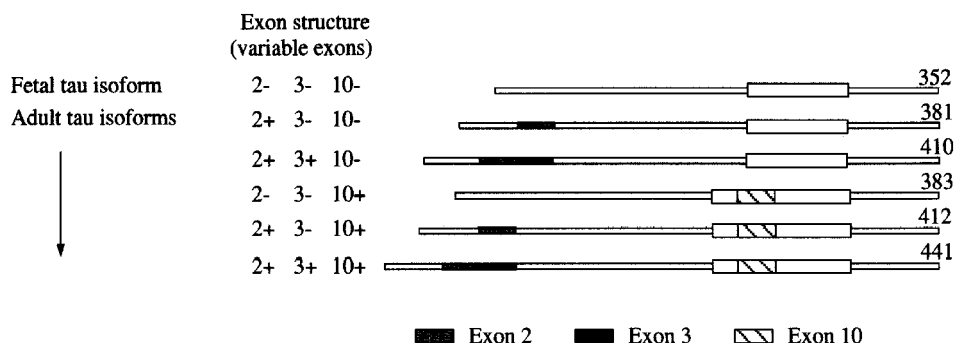


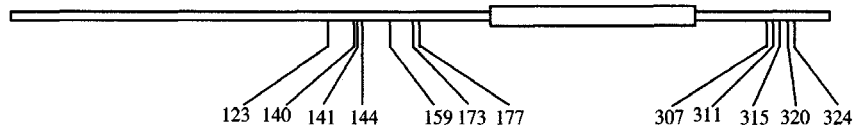
Figure 1. Schematic representation of human tau isoforms. Fetal and adult isoforms of tau and adult-specific exons are shown. The microtubule-binding domain in the carboxyterminal half of the protein is indicated by the open box. Note that one of the exons (exon 10) codes for an additional repeat and that the expression of exon 3 requires the presence of exon 2. The high molecular weight species of tau in peripheral nerves is not shown. This isoform contains two additional exons (4A and 6; Andreadis *et al.*, 1992).

(Weingarten *et al.*, 1975; Cleveland *et al.*, 1977b). Molecular cloning revealed that the tau proteins are produced from a single gene by alternative splicing giving yield to six different polypeptide chains with apparent molecular weights between 50 and 70 kDa and a high molecular weight tau isoform which is only expressed in neurons from the peripheral nervous system (PNS) (Figure 1; Goedert *et al.*, 1991; Andreadis *et al.*, 1992).

A striking feature of tau's primary structure is the presence of three or four imperfectly repeated stretches of 31 or 32 residues located in the carboxyterminal half of the protein. These repeats are also found in two other MAPs—MAP4 and MAP2—and constitute the core of tau's microtubule interacting unit (Lee *et al.*, 1989). Three of the longer isoforms contain an exon which codes for an additional repeat sequence thus potentially increasing tau's affinity to microtubules. Tau is a highly asymmetric protein with an axial ratio of 20:1 (Cleveland *et al.*, 1977a) and a length of about 35 nm (Mandelkow *et al.*, 1995) which probably accounts for its abnormally low mobility during polyacrylamide gelelectrophoresis in the presence of SDS (SDS-PAGE). In solution, tau behaves as if it were denatured with no evidence for compact folding and a minimal content of ordered secondary structure (alpha-helix or beta-sheet) (Schweers *et al.*, 1994). Consistent with its structure, tau is highly resistant to denaturation and survives short boiling and perchloric acid treatment.

*Correspondence: Dr. Roland Brandt, Department of Neurobiology, University of Heidelberg, Im Neuenheimer Feld 345, D-69120 Heidelberg, Germany; Tel: +49 (6221) 548329; Fax: +49 (6221) 544496; E-mail: brandt@sun0.urz.uni-heidelberg.de

A



B

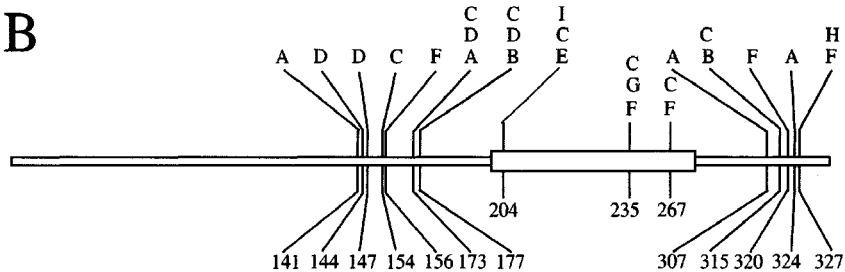


Figure 2. *In vivo* and *in vitro* phosphorylation of tau. A. Schematic representation of the position of sites which are phosphorylated in fetal rat brain tau (Watanabe *et al.*, 1993). For comparison, the position of the residues has been adjusted to the fetal human tau sequence. B. Phosphorylation of fetal human tau as determined from *in vitro* reactions using purified kinases. Note that most phosphorylation sites are clustered in two regions which flank the microtubule-binding domain. Abbreviations are: A: GSK-3 β (identical to tau protein kinase I; Ishiguro *et al.*, 1992; Ishiguro *et al.*, 1993); B: GSK-3 α (identical to protein kinase FA; Yang *et al.*, 1993); C: GSK-3 α after heparin activation (Yang *et al.*, 1994); D: PDPK (p34cdc2/p58cyclin A; Vulliet *et al.*, 1992); E: 35/41 kD kinase (Biernat *et al.*, 1993); F: PKA (Scott *et al.*, 1993b); G: PKC (Correas *et al.*, 1992); H: Ca²⁺-calmodulin dependent protein kinase (Steiner *et al.*, 1990); I: Phosphorylase kinase (Paudel, 1997).

Further complexity to the isoform pattern of brain isolated tau is added by phosphorylation. *In vivo*, tau is phosphorylated at several serine and threonine residues and can *in vitro* be phosphorylated by a variety of kinases involved in signal transduction mechanisms. These include cAMP-dependent protein kinase (PKA), protein kinase C (PKC), as well as growth factor-activated kinases such as proline-directed protein kinases (PDPK) (Figure 2). Phosphorylation at some sites regulates structural parameters of tau in that they induce conformational changes which result in lower electrophoretic mobilities during SDS-PAGE and increased tau stiffness (Hagestedt *et al.*, 1989). The stoichiometry of tau phosphorylation is higher in fetal than in adult brain (Kenessey and Yen, 1993; Watanabe *et al.*, 1993) indicating that the overall extent of tau phosphorylation is developmentally regulated.

In addition to phosphorylation, tau is glycosylated at serine and threonine residues (O-glycosylation). Bovine brain isolated tau contains single N-acetylglucosamine residues (GlcNAc) at more than 12 different sites with an average stoichiometry of at least 4 mols GlcNAc per mol of tau (Arnold *et al.*, 1996). O-glycosylation is a common feature of many cytoplasmic phosphoproteins and appears to be reciprocally related to phosphorylation. Like phosphorylation, O-glycosylation may serve as a regulatory modification (Hayes and Hart 1994).

Isoform Switches and Axonal Localization

Initially, tau was considered to be a neuron-specific MAP. Later it became clear that other brain cells such as astrocytes and perineuronal glia cells also express tau protein in considerable amounts (Papazosomenos and Binder 1987). In fact, recent data suggest that tau is rather ubiquitously expressed in a number of different tissues (Gu *et al.*, 1996) which agrees with the lack of neuronal specificity signals in the gene's promoter region (Andreadis *et al.*, 1996). However, the statement that tau is a neuronal MAP is still somehow valid since tau is clearly enriched in neurons.

Tau is expressed in all types of neurons with its level being higher in small caliber axons, i.e. mossy fibers in the hippocampus, than in large caliber axons (i.e. fasciculi gracilis and cuneatus in posterior columns) (Papazosomenos and Binder 1987). The pattern of the expressed isoforms changes during brain development. In the rat central nervous system (CNS), tau expression begins at embryonic day 13 with exclusively the shortest isoform (fetal tau) being expressed. During postnatal development, a transition to the expression of all low molecular weight isoforms occurs (Francon *et al.*, 1982) with a decline of fetal tau expression after postnatal day 8 (Kosik *et al.*, 1989). PNS neurons express a high molecular weight species of tau which contains two additional exons (Andreadis *et al.*, 1992) in addition to the low molecular weight tau isoforms.

In situ, tau is enriched in axons (Binder *et al.*, 1985; Brion *et al.*, 1988; Trojanowski *et al.*, 1989). It should however be noted that studies using different monoclonal tau antibodies came to slightly differing results with respect to tau's intracellular localization (Papazosomenos and Binder, 1987) and that tau could also be detected in the somatodendritic compartment. This is probably due to the fact that the antibody which was used in the original study is phosphorylation sensitive and that axonal tau is less phosphorylated than somatodendritic tau at least with respect to the epitope recognized by this antibody (Tau-1; see Figure 4). A different extent of tau phosphorylation in various subcellular compartments has recently also been confirmed in neuronal culture, where axonal tau was found to be less phosphorylated than tau in the somatodendritic compartment (Mandell and Banker 1996).

Several mechanisms may contribute to the axonal localization of tau. These include a selective transport of tau mRNA to the proximal axon (Litman *et al.*, 1993), locally regulated stability or microtubule binding (Kanai and Hirokawa 1995; Hirokawa *et al.*, 1996), and tau binding to an axonal plasmamembrane component (Brandt *et al.*, 1995; Kempf *et al.*, 1996). However studies on tau localization are hampered by the fact that the axon-specific distribution of tau which was originally observed *in situ* cannot be reproduced in many neuronal culture systems where tau is ubiquitously present. Interestingly, using a mild extraction protocol developed to retain intracellular interactions, tau is not only enriched in the axons of cultured neurons similar to its distribution *in situ* but exhibits a proximo-distal gradient with its concentration being highest in the distal axon (Figure 3B; Kempf *et al.*, 1996). Distal axonal tau binding occurs early in the development of polarity just at the time when one of the minor neurites begins to develop toward an axon suggesting a role for tau in determining axonal identity.

Microtubule Binding and Beyond

Tau was originally isolated because it interacts with microtubules and consequently classified as a “microtubule-associated protein”. In cell free assays, purified tau strongly promotes *de novo* microtubule nucleation and elongation in a concentration range similar to cellular tau and tubulin concentrations (Murphy *et al.*, 1977; Brandt and Lee 1993a, 1993b). Video-microscopic analysis has shown that tau promotes net microtubule assembly by altering growth, catastrophe, and shrinkage rates of microtubules (Drechsel *et al.*, 1992; Trinczek *et al.*, 1995; Drechsel 1997). Tau's repeat domain is the basic microtubuleinteracting unit, however sequences flanking the repeats considerably increase tau's microtubule binding and are required for an efficient *de novo* microtubule assembly with a centrally located proline-rich region being of particular importance (Figure 3 A; Brandt and Lee, 1993b; Gustke *et al.*, 1994; Goode *et al.*, 1997).

Tau's microtubule assembly promoting activity is sensitive to its phosphorylation state. Phosphorylation of serine 262 (all numbers refer to the longest low-molecular weight human tau isoform containing 441 residues; Figure 1) which is located within the repeat domain, completely abolishes tau's binding to microtubules (Drewes *et al.*, 1995). Phosphorylation of sites within the proline-rich region reduce tau's capacity to promote *de novo* nucleation of microtubules in cell-free assembly reactions but do not have a major effect on tau's microtubule binding and growth promoting activity (Brandt *et al.*, 1994; Léger *et al.*, 1997). This suggests that tau's role in microtubule assembly is modulated by the phosphorylation state of individual residues which may serve as “switches” to temporally and spatially regulate neuronal microtubule assembly.

In agreement with the *in vitro* data, tau associates with microtubules in cultured cells and promotes microtubule assembly when transfected into a variety of different cell types (for a review see Lee, 1997). The expression of tau in insect cells results in the formation of long, untapered processes which resemble axons (Knops *et al.*, 1991). In contrast, tau expression in mammalian cells is not sufficient to produce neurites. In transfected cells, processes are only formed when actin filaments are concomitantly disrupted by drug treatments (Edson *et al.*, 1993; Léger *et al.*, 1994, 1997). This suggests a more complex regulation of process formation in mammalian cells where, in addition to the promotion of microtubule assembly and stability, mechanisms for integrating other cytoskeletal elements may be involved. Interestingly, tau expression in transfected cells does not produce a stable microtubule array but induces only a partially stable but still dynamic state of microtubules (Kaech *et al.*, 1996). This property may be relevant for tau's role in the distal axon where, despite the high tau concentration, microtubules are highly dynamic (Ahmad *et al.*, 1993; Black *et al.*, 1996; Kempf *et al.*, 1996).

Antisense studies have implicated a role for tau in the development of polarity of cultured neurons (Cáceres and Kosik, 1990; Cáceres *et al.*, 1991). Most likely, this function is mediated by tau's aminoterminal projection domain which protrudes from the microtubule surface and does not contain sequence homologies with the dendritic MAP2. Sedimentation assays revealed a role for tau's projection domain in spacing microtubules apart (Brandt and Lee, 1993b, 1994). Indeed, when tau or MAP2 are expressed in insect cells, the resulting intermicrotubule distance closely reflects the characteristic spacing of microtubules in the axonal and dendritic compartment, respectively (Chen *et*

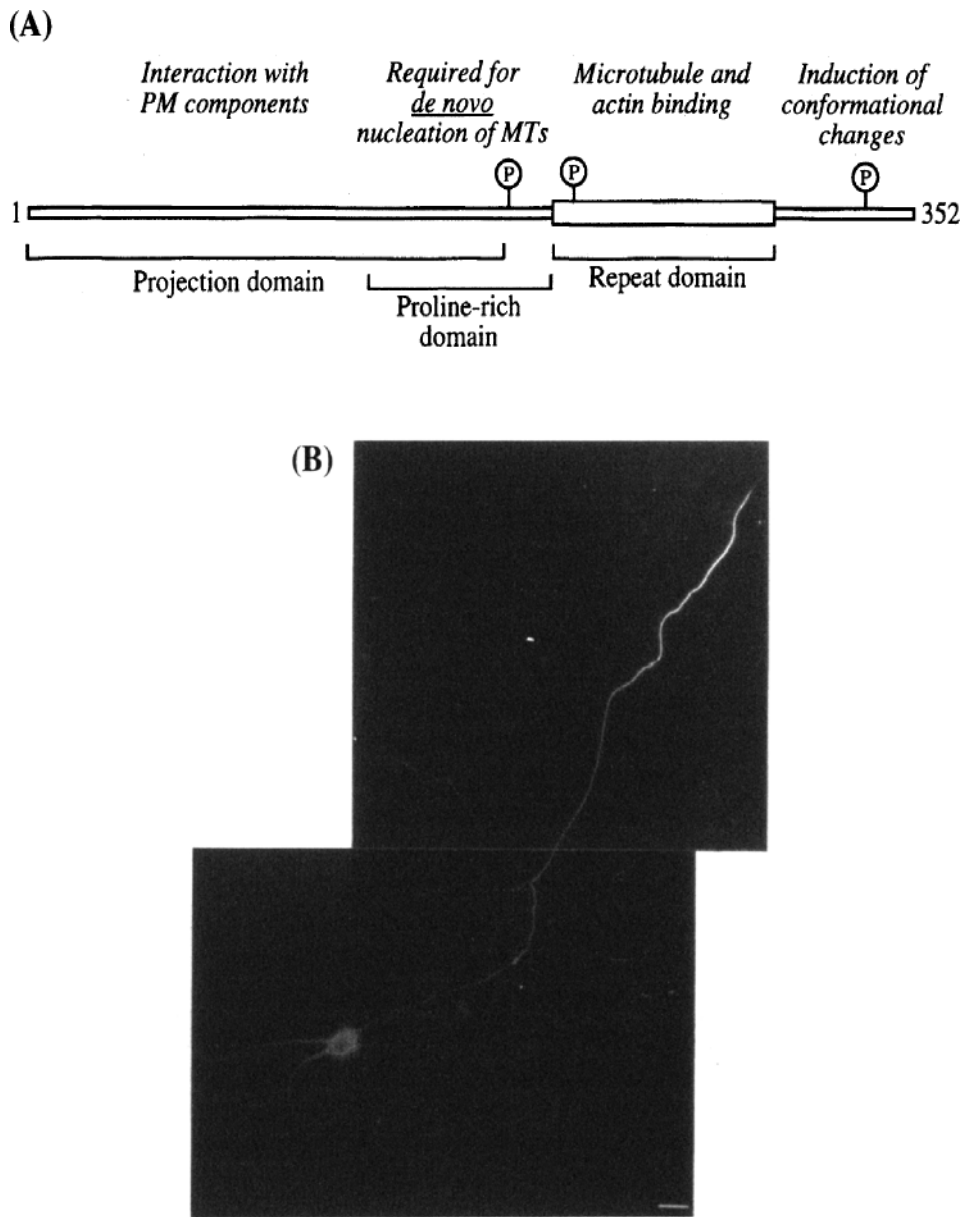


Figure 3. Functional organization of tau and distribution of tau binding in cultured human model neurons. A. Schematic representation of the functional organization of tau as determined from deletion studies (see text for references). Phosphorylation sites which may have a role in regulating tau's activities are indicated. Phosphorylation of serine 214 reduces tau's activity to induce *de novo* microtubule nucleation (Brandt *et al.*, 1994; Léger *et al.*, 1997), phosphorylation of serine 262 abolishes tau's microtubule binding, and phosphorylation of serine 416 induces a reduced electro-phoretic mobility during SDS-PAGE (Steiner *et al.*, 1990; Léger *et al.*, 1997). Abbreviations are: PM, plasmamembrane; MT; microtubule. B. Distribution of tau in terminally differentiated, polar human model neurons (NT2-N cells). The neurons were cultured and differentiated *in vitro* essentially as described earlier (Pleasure *et al.*, 1992) and fixed using a combined fixation-extraction protocol. Immunostaining employed monoclonal anti-tau (Tau-1) and rhodamine-coupled anti-mouse secondary antibody. Note that tau is enriched in the distal portion of the longest process which morphologically represents the axon. Scale bar, 10 μ m.

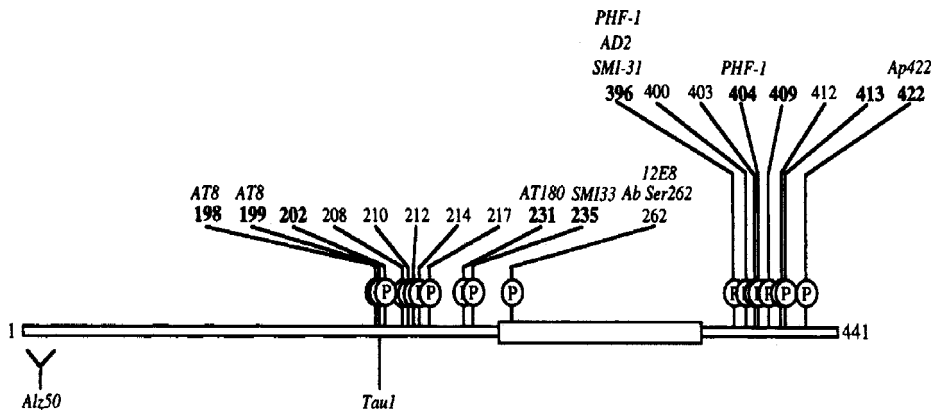


Figure 4. Schematic representation of the phosphorylation sites in PHF-tau and epitopes of commonly used tau antibodies. The positions of the phosphorylation sites in the longest human low molecular weight tau isoform containing 441 residues (see Figure 1) as determined by (Morishima-Kawashima *et al.*, 1995) are indicated. Major phosphorylation sites are indicated in bold. Commonly used tau antibodies are indicated in italics. Most of the shown antibodies are phosphate dependent with the exception of Alz50 which recognizes a PHF-tau specific conformation at Arg5-Phe8. Tau-1 requires a dephosphorylated epitope at position 131–149 (Liu *et al.*, 1993b; Szendrei *et al.*, 1993) (for reference see Ksiezak-Reding *et al.*, 1997).

al., 1992). Most likely, the spacer activity of tau and MAP2 reflects a steric but not a sequence-specific effect of the projection domain.

Evidence for a specific role of tau's projection domain in neurons comes from experiments where tau deletion fragments were expressed in neural cells (rat PC12). In transfected cells, tau interacts with neural plasmamembrane components through its aminoterminal non-microtubule-binding projection domain (Brandt *et al.*, 1995) which suggests a role for tau as a mediator of microtubule-plasmamembrane interactions during the development of neurites. Consistent with this view, overexpression of a tau fragment lacking the micro-tubule-binding domain suppresses process formation in NGF-treated PC12 cells (Brandt *et al.*, 1995) indicating that the deletion fragment competes for a non-microtubule binding site which is required for process formation. The molecular identity of the axonal plasmamembrane components with which tau interacts is still unknown but appears to involve a complex interaction between different cytoskeletal elements (Kempf *et al.*, 1996) and to be regulated by signal transduction mechanisms (Brandt *et al.*, submitted).

The function of tau in neurons is still unclear. Tau may have a role in translating transient actin-based filopodial movements into the microtubule reorganization which finally determines axonal directionality or, alternatively, it may have a role in anchoring axonal membrane components. Consistent with the latter hypothesis, it has been found that an actin-associated growth cone component depends on tau for its localization (DiTella *et al.*, 1994).

Given the result that tau expression is required for axonal development in culture, one would expect an essential role of tau in neuronal development. It came therefore as a surprise that mice lacking tau develop normally and show only minor neuronal abnormalities (Harada *et al.*, 1994). In these mice, backup mechanisms may exist which substitute for the function of tau. Indeed, another neuronal MAP

—MAP1A—is upregulated in tau knockout animals however the functional significance of this finding is unclear.

It should be noted that tau not only qualifies as a microtubule-associated protein but can be also considered as an actin-binding protein (Griffith and Pollard 1978, 1982). The interaction of tau with actin filaments is phosphorylation sensitive (Selden and Pollard, 1983), however, the physiological significance of the tau-actin association is unclear since tau clearly colocalizes with microtubules in cells. In addition, the tau domain which interacts with actin appears to be the same as the microtubule-binding domain (Correas *et al.*, 1990) thus excluding a potential role of tau as an actin-microtubule linker.

Taken together, the functional data suggest that tau serves different functions which may be independently regulated by phosphorylation. [Figure 3](#) summarizes the results on the functional organization of tau and indicate possible regulatory mechanisms which may be involved in modulating tau's cellular activities.

TAU IN DISEASE

Filaments and Tangles

In the brain of subjects affected with AD, NFTs accumulate as a characteristic histopathological feature. At least three distinct types of NFTs can be distinguished (Bondareff *et al.*, 1994): Type 1 NFTs consist of intraneuronal argyrophilic inclusions which are formed in the cell body of subsets of pyramidal neurons and from which they extend into the proximal dendrites. After neuronal cell death, type 2 NFTs as extracellular but yet compact deposits form which then swell as they are progressively degraded by astrocytes to become type 3 NFTs.

NFTs contain abnormal tau-immunoreactive filaments (paired helical filaments, PHFs) as their core and major component (Brion *et al.*, 1985; Grundke *et al.*, 1986; Pollock *et al.*, 1986). Ultrastructurally, PHFs consist of double- or single-stranded ribbons twisted every 70–90 nm with a maximal width ranging between 15 and 25 nm. In addition to the twisted PHFs, straight filaments which are narrower (width between 12 and 18 nm) often coexist in the same brain. Straight filaments may be an intermediate of twisted PHFs or a result from ultrastructural instability (Ksiezak-Reding *et al.*, 1996).

In the early 90s, several methods have been developed to isolate and analyze the molecular constituents of PHFs (for a summary see Ksiezak-Reding *et al.*, 1997). The procedures take advantage of the stability of filaments in the presence of 0.8 M NaCl, their insolubility in Sarcosyl (N-lauroyl-sarcosine), and their differential solubility in SDS which allows separation of a SDS-soluble from a SDS-insoluble fraction of PHFs. This procedure makes it possible to analyze purified PHFs as filaments using ultrastructural approaches. In addition, biochemical analysis of soluble PHF-tau for molecular components and posttranslational modification can be performed.

When separated by SDS-PAGE, SDS-soluble PHFs consist primarily of three tau-immunoreactive bands of molecular masses of 60, 64 and 68 kDa. In the SDS-insoluble PHFs, non-tau material including a ferritin polypeptide and a proteoglycan fraction is present in addition to low molecular weight tau isoforms and a covalently crosslinked tau polymer with high molecular mass. SDS-soluble

as well as SDS-insoluble fractions show immunoreactivity with antibodies against phosphorylated and non-phosphorylated tau epitopes. Compared to tau isolated from autopsy material, PHF-tau is 3–4 times more phosphorylated (Kenessey and Yen, 1993). Antibody accessibility and proteolysis studies of purified PHFs suggest that the microtubule-binding domain is blocked in the core of the filament by the more peripherally located amino- and carboxyterminal regions.

The proportion of tau isoforms in PHF-tau and in a tau fraction from normal brain is different. In particular, exon 3 is overrepresented in PHF-tau whereas exon 2 is decreased as judged by immunoblotting and enzyme-linked immunosorbent assay (ELISA) experiments (Liu *et al.*, 1993a). The reason for these differences is unclear because tau transcripts which contain exon 3 usually contain exon 2 as well (Figure 1; Andreadis *et al.*, 1992); the different immunoreactivity may be indicative for a proteolytic degradation of tau's extreme amino terminus. Exon 10 which codes for an additional repeat in tau's microtubule binding domain is reduced in PHF-tau as compared to normal adult tau (Ksiezak-Reding *et al.*, 1995).

Tau Modification in Disease Brain

The development of methods for the analysis of PHF-tau made a detailed analysis of posttranslation modifications possible and four major types of modifications—phosphorylation, glycation, racemization, and ubiquitination—have been identified and characterized to date (Table 1).

The total phosphate content of PHF-tau (6–8 mol phosphate/mol tau) is significantly higher than the phosphate content of tau obtained from autopsy material (1.9 mol phosphate/ mol protein) (Kenessey and Yen, 1993). It has later been shown, that tau isolated from PHFs is phosphorylated at many of the same sites as tau from freshly biopsied material suggesting that much of the observed differences are not caused by different activities of kinases but may be due to a lack of phosphatase activity during postmortem processing in AD brain (Matsuo *et al.*, 1994). Phosphopeptide analysis revealed the presence of 19 phosphorylated sites in PHF-tau (Figure 4A; Morishima-Kawashima *et al.*, 1995). 15 sites contain serine residues and four sites threonine residues. Proline-directed as well as non proline-directed sites are involved. Not all of the 19 sites are fully phosphorylated and 10 major phosphorylation sites have been identified. The comparison with the sites which are phosphorylated by purified kinases *in vitro* reveals, that none of the known kinases is able to phosphorylate all sites alone. Glycogen synthase kinase-3 (GSK-3 α and GSK-3 β), cyclin-dependent kinase 5 (cdk5), CDC2, mitogen-activated protein kinase (MAPK), PKA, and mark p110 are each able to phosphorylate some of the sites shared by PHF-tau (compare to Figure 2B). Substrate modulation (Brandt *et al.*, 1994) or prior phosphorylation by other kinases can increase the phosphorylation of tau and multiple kinases may act in sequence to phosphorylate tau to the state observed in PHF-tau.

The sites which are phosphorylated in PHF-tau are not uniformly distributed but are clustered in two regions of tau. Half of the major phosphorylation sites are localized in the proline-rich region which amino-terminally flanks tau's microtubule binding domain and the other half are localized at tau's extreme carboxyterminus.

Table 1. Posttranslational modifications of tau in AD.

<i>Modification</i>	<i>Characterization</i>	<i>Implication</i>	<i>Reference</i>
Phosphorylation	Covalent binding of phosphate on serine- and threonine-residues.	Regulation of MT binding (Ser262) and MT nucleation. Changes of paracrystal structure. Inhibition of self assembly and degradation.	Drewes <i>et al.</i> 1995 Léger <i>et al.</i> 1997 Hagestedt <i>et al.</i> 1989 Guttmann <i>et al.</i> 1995 Litersky and Johnson 1992
Glycosylation	Covalent binding of GlcNAc on serine- and threonine-residues.	Regulation of MT binding. No glycosylation of isolated PHFs.	Arnold <i>et al.</i> 1996 Ledesma <i>et al.</i> 1994 Wang <i>et al.</i> 1996a
Glycation	Covalent binding of reducing sugars on lysine residues (Non-enzymatic reaction). Further complex modification results in advanced glycation end products (AGE).	Glycation of tau reduces binding to microtubules <i>in vitro</i> . Glycation of PHF-tau <i>in vitro</i> increases aggregate formation. Glycated products have a reduced solubility.	Ledesma <i>et al.</i> 1994 Ledesma <i>et al.</i> 1995
Racemation	Racemation of L-aspartate to D-aspartate. Observed in several long-lived proteins.	Increased resistance to proteolysis.	Yang and Ksiezak-Reding 1995; Bada 1985
Ubiquitination	Covalent binding of ubiquitin. Monoubiquitination and multi-chained ubiquitination are observed.	Ubiquitination mostly found in extracellular NFTs. Multubiquitination (strong degradation signal) comprise 20–30%. Monoubiquitination may serve not as a strong degradation signal in PHFs.	Mori <i>et al.</i> 1987 Morishima <i>et al.</i> 1993
Degradation	Proteolytically processed by proteases	Truncated and non truncated forms are found in NFTs. If truncation is primary or secondary event is unclear.	Kondo <i>et al.</i> 1988

Another characteristic feature of PHF-tau is the modification of lysine-residues by non-enzymatic protein glycation (for a recent review see Harrington and Colaco, 1997). Tau is rich in lysine residues which constitute about 10% of the amino acid content and glycation probably is the reason that PHF-tau contains 35% less lysine than normal tau (Liu *et al.*, 1991). Glycation involves a complex network of reactions where reducing sugars initially react with the ϵ -amino group of lysine to form a reversible condensation product (Schiff base). This reaction is followed by a series of nearly irreversible rearrangements and subsequent advanced glycation reactions which yield different covalent adducts often termed advanced glycation end-products (AGEs). Both, the intracellular and extracellular NFTs show AGE immunoreactivity (Smith *et al.*, 1994; Yan *et al.*, 1994). In tau, glycation is highest within the second-last repeat (Ledesma *et al.*, 1995).

Comparative analysis of PHF-tau and normal tau revealed that PHF-tau contains a higher D-aspartate content compared to normal adult brain tau (4.9 versus 2.8%; Kenessey *et al.*, 1995). Racemization of aspartic acid has been reported in several long-lived proteins (Bada 1985) and may not be a modification specific for PHF-tau but simply a consequence of the higher proteolytic stability of PHF-tau compared to normal tau (Mercken *et al.*, 1995).

As another posttranslational modification, ubiquitin has been identified as a component of the SDS-insoluble PHFs (Mori *et al.*, 1987; for a review see Mori, 1997). Protein chemical analysis of PHF-tau revealed a high ubiquitin content with multi-chained ubiquitin, which usually serves as a degradation signal for a 26S proteasome complex (for a review see Herskho and Ciechanover, 1992). Multi-chained ubiquitin comprises about 20–30% of the total ubiquitin in PHF (Morishima-Kawashima *et al.*, 1993). Ubiquitination of various intraneuronal inclusions is not unusual and appears to occur in many neurodegenerative diseases (for a review see Lowe *et al.*, 1993). Despite their ubiquitin content, PHFs are stable and accumulate in neurons. This may be due to the high content of the monoubiquitinated form which may not serve as a degradation signal. Alternatively or in addition, the proteasome complex may fail to access the ubiquitinated sites in PHFs.

Staging Tau Pathology

NFTs develop in a characteristic sequence during AD in very limited brain regions. Several stages with a progressive increase in cortical destruction have been distinguished (Braak *et al.*, 1993). First changes are seen in the entorhinal cortex from which the destructive process then spreads into the hippocampal formation and eventually the isocortex.

Correlating the histopathological stages with the staining for phosphorylation-specific tau antibodies has lead to a grouping of neurons with increasing abnormality (Braak *et al.*, 1994): Group 1 neurons appear normal, are devoid of argyrophilic NFTs but are ubiquitously stained with the phosphorylation sensitive antibody AT8 often used to identify NFTs (see [Figure 4](#) for the epitopes of frequently used tau antibodies). Group 2 neurons show first changes in the morphology of their neurites in that the distal portions of the dendrites are curved and show thickened portions. In the thickened portions often rod-like inclusions are stained both by silver as well by the AT8 antibody. Group 3 neurons display more pronounced alterations in their dendritic morphology which contain neuropil threads, and NFTs in the soma. In later stages (group 4 and group 5 structures), the fibrillary material becomes extracellular and negative for AT8 immunoreactivity.

Sequential changes in tau immunoreactivity during the development of PHFs have also been observed with other phosphorylation-sensitive antibodies. Tau is phosphorylated in a pretangle state in neuropil threads at serines 199, 202 and 409. At the stage of PHFs tau is additionally phosphorylated at serine 396/404 and threonine 231. In extracellular NFTs, tau is mainly phosphorylated at serine 396/404 (Kimura *et al.*, 1996). Site-specific antibodies have also been used to characterize the different types of NFTs (Bondareff *et al.*, 1994). Intracellular tangles are immunoreactive against antibodies recognizing epitopes throughout the tau molecule. In contrast, compact and dispersed extracellular tangles show less immunoreactivity against antibodies recognizing tau's aminoterminal.

Taken together, the results indicate that changes in the immunoreactivity of tau precede the formation of argyrophilic NFTs and neuropil threads. Besides its diagnostic value, it is however not clear whether the phosphorylation of tau at sites which are not or to a lower extent phosphorylated in normal tau is a cause rather than an unrelated side-effect of tau aggregation. The further development of neurofibrillary degeneration is then characterized by a sequential stripping of PHFs when they become extracellular resulting in the loss of amino-terminal and phosphorylated epitopes.

Functional Implications

A characteristic feature of tau in PHFs is a high phosphorylation state of several sites. Since tau's activity on microtubule assembly is phosphorylation-sensitive, phosphorylation of tau in AD may result in a loss of tau's activity to promote microtubule assembly and stability. This may contribute to the breakdown of axonal microtubules and to the impairment of axonal transport as it has been reported in affected neurons in AD brain (Dustin and Flament-Durand, 1982).

In fact, tau which has been isolated from AD brains (AD-tau) induces a slower polymerization and a lower steady state of microtubule assembly when compared to tau isolated from nondemented age-matched control brains (Lu and Wood 1993). Consistently, in similar assays, PHF-tau is almost assembly incompetent compared to fetal tau (Yoshida and Ihara 1993). Microtubule assembly competence could be restored in AD-tau and PHF-tau after phosphatase treatment (Alonso *et al.*, 1994; Iqbal *et al.*, 1994; Garver *et al.*, 1996; Wang *et al.*, 1996b) indicating that tau's activity is reversibly suppressed by phosphorylation and not by any other type of posttranslational modification. *In vitro* phosphorylation of tau by kinases which phosphorylate sites that are modified in PHF-tau results in a decrease of tau's activity to promote microtubule nucleation and growth (Scott *et al.*, 1993b; Brandt *et al.*, 1994).

Interestingly, AD-tau not only has little activity in promoting microtubule assembly but also inhibits microtubule assembly when added to normal tau (Alonso *et al.*, 1994) or high-molecular weight MAPs (Alonso *et al.*, 1997). This suggests that an AD-like phosphorylation of tau could contribute to a breakdown of microtubules in affected neurons through two mechanisms: firstly, because modified tau has little activity on microtubule assembly by itself, and secondly, because it sequesters normal tau or other MAPs thereby amplifying the destabilization effect.

It is not clear whether the phosphorylation of individual sites has a critical role in changing tau's activity on microtubule assembly during AD or whether the loss of tau's activity is the result of an overall increase of the stoichiometry of tau phosphorylation. A potentially important site could be serine 262 which is located within tau's microtubule binding domain and whose phosphorylation

drastically reduces tau's interaction with microtubules (Drewes *et al.*, 1995). Serine 262 is phosphorylated in human tau from fetal, adult, and AD brains (Seubert *et al.*, 1995). However, recently it has been shown that serine 262 is not a major phosphorylation site *in vivo* and little or no difference between PHF-tau and normal tau in the extent of phosphorylation at this site is observed (Liu and Yen, 1996). Most of the residues which are modified by phosphorylation in PHF-tau are located in two regions which aminoterminally and carboxyterminally flank tau's microtubule binding domain (see Figure 4). Using site-directed mutagenesis it has been shown that individual sites within the carboxyterminal region critically affect tau's conformation whereas sites in the aminoterminal region regulate tau's activity on microtubule assembly (Léger *et al.*, 1997). It will be interesting to test the effect of mutated PHF-sites in similar experiments.

In addition to its effect on microtubule assembly, phosphorylation of tau may also affect its stability against proteolytic degradation. Phosphorylation by PKA inhibits the degradation of tau by calpain *in vitro* (Litersky and Johnson, 1992), and experimentally induced hyperphosphorylation in cells reduces tau turnover (Vincent *et al.*, 1994). Thus, phosphorylation-induced stabilization may contribute to tau accumulation thereby amplifying the sequestering effect of modified tau.

Tau glycation may also contribute to tau's loss of activity in promoting microtubule assembly. Since the main target is the microtubule binding domain, glycation may directly inhibit tau's microtubule binding (Ledesma *et al.*, 1995). More indirectly, glycation reduces the solubility of proteins and facilitates aggregation which could contribute to the sequestration of tau from microtubules (Ledesma *et al.*, 1994, 1996).

Phosphorylation and other posttranslational modifications may also affect functions of tau other than those on microtubules. Tau binds to neural plasmamembrane components through its aminoterminal non-microtubule binding domain and this interaction may be important for tau's axon-specific localization (Brandt *et al.*, 1995; Kempf *et al.*, 1996). In fact, the binding of tau to the distal axon appears to be sensitive to the phosphorylation of sites which are modified in PHF-tau (Brandt *et al.*, submitted). An AD-like phosphorylation may cause a relocalization of tau from the axon to the cell body thus contributing to a loss of tau from axonal microtubules and increasing tau accumulation in the cell body.

TAU'S ROLE IN AD PATHOLOGY

Cell-free Models

The development of models for tau pathology has focused on two aspects: on the reproduction of the formation of fibrillar tau aggregates similar to PHFs, and on the identification of pathogenic mechanisms which influence tau pathology.

Recently, tau aggregates which morphologically closely resemble PHFs have been produced in cell free assembly reactions. These experiments used recombinant fetal tau which has been incubated in the presence of sulphated glycosaminoglycans such as heparin or heparan sulphate (Goedert *et al.*, 1996). Interestingly, earlier studies have shown that heparin increases tau phosphorylation *in vitro* (Brandt *et al.*, 1994), although phosphorylation of tau had no effect on fibril formation under these conditions. This

suggests that hyperphosphorylation of tau accompanies fibril formation but does not have a causative role for aggregation at least *in vitro*. In the same study it has been shown that heparan sulphate and hyperphosphorylated tau coexist in nerve cells of AD brains at early stages of neurofibrillary pathology indicating that sulphated glycosaminoglycans may be a key factor in the formation of PHFs.

Tau aggregation involves the microtubule-binding domain and truncated tau fragments which contain this domain propagate aggregation (Wischik *et al.*, 1996). The development of such cell-free aggregation assays allows to analyze for factors which may contribute to PHF formation and to screen for drugs which are active in preventing tau self-assembly. In fact, besides sulphated glycosaminoglycans, aluminium salts (Scott *et al.*, 1993a), RNA (Kampers *et al.*, 1996), oxidation of cysteine-residues (Schweers *et al.*, 1995) and transglutaminase activity (Dudek and Johnson, 1993) have been shown to affect tau aggregation. As a candidate for a drug which prevents PHF formation, a nonneuroleptic phenothiazine has been identified (Wischik *et al.*, 1996). Phenothiazine blocks tau-tau binding interaction through the repeat domain which is involved in tau aggregation but does not affect the tau-tubulin interaction, which also occurs through the same domain.

Interestingly, apolipoprotein E (ApoE) isoforms differentially interact with tau's micro-tubule binding domain (Strittmatter and Roses, 1995; Roses *et al.*, 1996) in a phosphorylation-dependent manner (Strittmatter *et al.*, 1994; Huang *et al.*, 1995). It is conceivable that this interaction affects tau-tau binding. This may provide a functional link between the ApoE $\epsilon 4$ allele as a known risk factor for AD (for a review see Mahley *et al.*, 1996) and tau pathology.

Cellular and Animal Models

To analyze the intracellular mechanisms which contribute to tau pathology it is important to develop appropriate cellular or animal models. Cellular models have been an important tool to analyze mechanisms which are involved in producing a hyperphosphorylation state of tau which may represent an early state in tau pathology (Table 2). For this purpose, conformation- and phosphorylation-specific tau antibodies are instrumental (Figure 4). A hyperphosphorylation state of tau as detected with some of these antibodies can be induced by activation or overexpression of GSK-3 β and PKC, but not of MAP kinase or MAP kinase kinase. In evaluating these experiments it should however be kept in mind, that in many of the experiments non-neuronal cells or neuroblastoma cells which may only poorly reflect terminally differentiated polar neurons were used and that "real" neurons may respond differently.

To test for the effect of other factors which may play a role in tau pathology, cultured cells were incubated with amyloid beta (A β), the main constituent of the amyloid plaques. A β is toxic and induces neurodegeneration in cultured rat brain cells (Yankner *et al.*, 1990) and differentiated neural cell lines (Lambert *et al.*, 1994). Treatment of cultured neurons with A β fibrils but not soluble A β increases tau phosphorylation converting tau to a soluble form which does not bind to microtubules and causes its accumulation in the somatodendritic compartment (Busciglio *et al.*, 1995). These changes in tau activity are due to phosphorylation since dephosphorylation restores microtubule binding. The effect of A β involves the inactivation of phosphatidylinositol-3 kinase and activation of GSK-3 β (also named tau protein kinase I (TPK-I)) (Takashima *et al.*, 1996). GSK-3 β may have a key

role in the pathogenic process since it is essential for A β induced neurotoxicity (Takashima *et al.*, 1993). Interestingly, another substrate for GSK-3 β is the mitochondrial enzyme pyruvate dehydrogenase whose inactivation by phosphorylation results in mitochondrial dysfunction and which may contribute to neuronal cell death (Hoshi *et al.*, 1996).

Also other extracellular factors induce changes in tau's phosphorylation state. The excitatory amino acid glutamate increases tau phosphorylation in primary neuronal culture models (Sautiere *et al.*, 1992; Sindou *et al.*, 1992; Sindou *et al.*, 1994), however also an increased dephosphorylation of tau has been reported (Davis *et al.*, 1995). Interestingly, A β increases glutamate-induced neuronal degeneration and calcium ionophore neurotoxicity suggesting that A β destabilizes neuronal calcium homeostasis and thereby renders neurons more vulnerable to environmental insults (Mattson *et al.*, 1992). Aluminium salts, which have long been implicated in neuronal cell death and which are a suspected risk factor for AD (for a review see Armstrong *et al.*, 1996), induce a change in tau staining (Kawahara *et al.*, 1992) but do not cause a degeneration of neurons (Mattson *et al.*, 1993). However, chronic exposure to aluminium salts may have different effects in combination with other insults. Interestingly, in cell free assays aluminium salts can induce a non-enzymatic phosphorylation of tau (Abdel-Ghany *et al.*, 1993) which may render the picture on tau modification more complex. Tau phosphorylation is also increased in neuroblastoma cell cultures by the extracellular matrix components fibronectin and laminin (Martin *et al.*, 1995) which suggests that antigenic changes in tau are a common feature during neuronal differentiation.

Overexpression of tau in cultured cells or transgenic mice does not lead to PHF formation although changes in tau phosphorylation and localization have been found. Transgenic mice which express human tau show AT8 immunoreactivity in dendritic processes which could represent an early event in tau pathology (Götz *et al.*, 1995). However, these experiments are difficult to interpret since the antigenic changes may be the result rather than the cause of tau's somatodendritic localization and may simply be caused by the "flooding" of the cells with tau.

Until now, the various cellular and animal models have allowed to identify factors and conditions which cause an increased phosphorylation at sites which are modified in tau from AD brain. However, no cell or transgenic model including mice which produce amyloid plaques has yet been identified which develops NFTs thus excluding a further analysis of the role of tau aggregation in the disease process.

Tau Modification: Common End Point or Causative Role in Disease?

NFTs clearly represent a histopathological hallmark of AD and antibodies against modified tau proteins have proven to be a valuable tool as a marker for AD pathology. However it is far from being clear which role tau plays in AD pathology. The formation of tau aggregates during disease process is not restricted to AD but also occurs in other neurodegenerative disorders including corticobasal degeneration, progressive supranuclear palsy and Pick's disease (Feany *et al.*, 1996). Tau aggregates have even been identified in the livers of patients with alcoholic hepatitis and corresponding mouse models where they contain abnormally phosphorylated tau isoforms (Kenner *et al.*, 1994; Zatloukal *et al.*, 1995). This suggests that tau aggregates do not play a role which is specific for AD.

Table 2. Animal and cellular models for tau pathology.

<i>Models</i>	<i>Experimental set-up</i>	<i>Observation</i>	<i>Reference</i>
Ageing animals			
cat		spontaneous diffuse A β deposits, no NFTs	Cummings <i>et al.</i> , 1996
dog		spontaneous diffuse A β deposits, no NFTs	Cummings <i>et al.</i> , 1996
bear		spontaneous diffuse A β deposits, PHF-1-positive neurofibrillary tangles	Tekirian <i>et al.</i> , 1996
primates		spontaneous A β deposits (dramatic differences between species), no NFTs	Martin <i>et al.</i> , 1991; Nakamura <i>et al.</i> , 1996; Heilbroner and Kemper 1990
sheep		NFT-like structures stained by silver impregnation and thioflavin-S, immunoreactive against tau (Alz 50 and AT8)	Nelson and Saper 1995
Transgenic mice			
APP	expression of APP751 (low copy)	no thioflavin or congo red deposits, no NFTs	Higgins <i>et al.</i> , 1995
	expression of APP717 (high copy)	fibrillar amyloid deposits, neurodegeneration, no NFTs	Games <i>et al.</i> , 1995
	expression of APP695sw (high copy)	amyloid deposits, memory deficits, no NFTs	Hsiao <i>et al.</i> , 1996
tau	expression of htau441	somatodendritic phosphorylated tau (PHF-1 and AT8)	Götz <i>et al.</i> , 1995
calcineurin A α	knock out of calcineurin A	accumulation of hyperphosphorylated tau (PHF-1, AT270 and AT180), abnormalities in cytoskeleton, deficits in learning and memory, no NFTs	Kayyali <i>et al.</i> , 1997

Table 2. (Continued).

<i>Models</i>	<i>Experimental set-up</i>	<i>Observation</i>	<i>Reference</i>
Cellular models			
brain cells culture (rat)	treatment with A β	neurodegeneration	Yankner <i>et al.</i> , 1990
fetal hippocampal neurons (rat)	treatment with A β	fibrillary A β induces phosphorylation of tau (PHF-1)	Busciglio <i>et al.</i> , 1995
		activation of GSK-3 β and inactivation of PI-3-Kinase	Takashima <i>et al.</i> , 1996
		GSK-3 β is essential for A β induced neurotoxicity	Takashima <i>et al.</i> , 1993
		GSK-3 β inactivates pyruvate dehydrogenase which may cause neuronal cell death	Hoshi <i>et al.</i> , 1996
primary neuronal culture (rat)	treatment with glutamate	tau phosphorylation (AT8)	Sindou <i>et al.</i> , 1994
		tau dephosphorylation (Tau-1 and AT8)	Davis <i>et al.</i> , 1995
cerebral cortical culture (human)	treatment with A β	increasing of glutamate induced neuronal degeneration and calcium ionophore neurotoxicity	Mattson <i>et al.</i> , 1992
cerebral cortex neurons (rat)	treatment with aluminium	change in tau staining	Kawahara <i>et al.</i> , 1992
		no induction of neurodegeneration	Mattson <i>et al.</i> , 1993
neuroblastoma culture (human)	culture on fibronectin and laminin	phosphorylation of tau (PHF-1)	Martin <i>et al.</i> , 1995
neuronal NT2N cells (human)	treatment with insulin and insulin like growth factor 1	phosphorylation of tau by insulin and insulin like growth factor 1 mediated through inhibition of GSK-3 β via PI-3-kinase/PK B signaling pathway	Hong and Lee 1997

Note that no animal or cell culture model develops PHFs or NTFs.

However, since—in contrast to amyloid plaque formation—the formation of tangles correlates closely with neuronal cell death, tangle formation may be a critical factor that kills the neurons. If true, this clearly would have important therapeutic implications. However, a proof for a direct role of tau in neuronal death is still missing and would require the development of cellular or animal models which develop tangles. An increased tau phosphorylation alone appears not to be lethal for neurons since a staining for AD-like phosphorylated tau isoforms can be detected very early in some adult brains without apparent neuronal death and is also present in tau overexpressing transgenic mice which do not show excessive neuronal death.

To date, a model for the role of tau in the development of AD is highly speculative but may help to phrase questions which need further experimental testing (Figure 5). An increased phosphorylation of specific residues in tau may be an early event in the formation of tau pathology. Culture and animal models point to an important role of GSK-3 β and the phosphatase calcineurin. Interestingly, GSK-3 β could also play an important role in A β induced neurotoxicity and mitochondrial malfunction which may link A β and tau pathology. Increased tau phosphorylation then could have important consequences which would pave the way for the development of tangles. Phosphorylation may reduce tau's binding to microtubules and may cause a relocalization of tau into the somatodendritic compartment and a decreased cellular tau turnover. As a result, tau may accumulate in the cell body where it aggregates into tangles with a possible involvement of other factors such as for example sulphated glycosaminoglycans. Newly formed aggregates may then sequester additional tau and other MAPs resulting in microtubule breakdown, weakening of cellular stability, and a disturbance of microtubule-dependent transport processes. Tau in PHFs may then be further modified and other proteins may coaggregate to form NFTs. As they grow, these NFTs may kill the already weakened neurons.

This model is compatible with most of the experimental results and suggests a role of NFTs as a common end point of diseases which involve tau pathology. It also suggests that an initial event in the formation of NFTs is a change in tau phosphorylation with all other modifications being secondary. In different diseases, the initial trigger could be very different and may involve various stressors which induce sudden changes in the complex equilibrium between the activities of multiple kinases, phosphatases, and the tau expression pattern. However, once triggered, neuronal cell death may be inevitable. It will be very important to test the predictions of such a model for the role of tau pathology also in view of a possible development of therapeutic drugs which could intervene with critical steps in tangle formation.

ACKNOWLEDGEMENTS

We thank Dr. Jeremy Garwood for critically reading the manuscript. This work was supported by a Heisenberg fellowship from the Deutsche Forschungsgemeinschaft (R.B.) and Sonderforschungsbereich 317 "Molekularbiologie neuraler Mechanismen und Interaktionen".

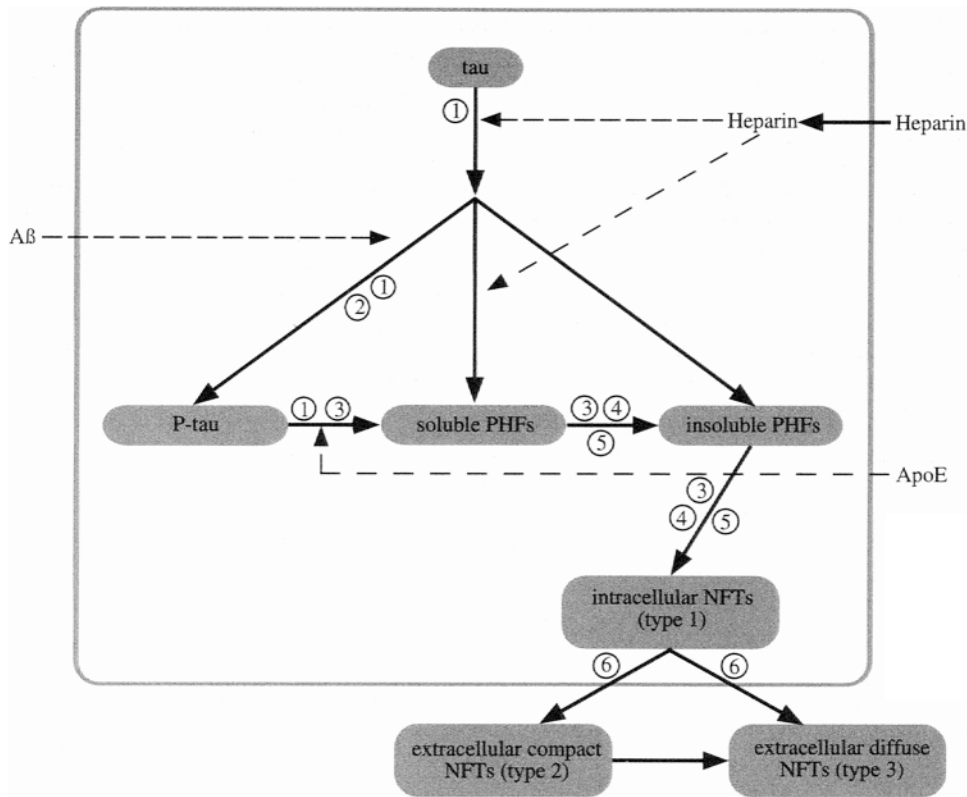


Figure 5. Hypothetical model for the intracellular events and extracellular factors involved in tau pathology. Critical events appear to be the formation of PHFs and NFTs which are accompanied by different posttranslational modifications. The role of tau phosphorylation in PHF formation, i.e. whether phosphorylation is required for tau aggregation or represents an independent side-effect, is unclear. Several extracellular factors may influence individual steps in the formation of tau aggregates. Heparin may enter the cell and influence tau phosphorylation as well as phosphorylation-independent tau aggregation, ApoE may influence tau-tau binding, and A β induces tau phosphorylation of sites which are modified in PHF-tau. 1: phosphorylation, 2: deglycosylation, 3: glycation, 4: racemization, 5: ubiquitination, 6: degradation.

REFERENCES

- Abdel-Ghany, M., el-Sebae, A.K., and Shalloway, D. (1993) Aluminium-induced nonenzymatic phospho-incorporation into human tau and other proteins. *J. Biol. Chem.*, **268**, 11976–11981.
- Ahmad, F.J., Pienkowski, T.P., and Baas, P.W. (1993) Regional differences in microtubule dynamics in the axon. *J. Neurosci.*, **13**, 856–866.
- Alonso, A.C., Zaidi, T., Grundke-Iqbal, I., and Iqbal, K. (1994) Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease. *Proc. Natl. Acad. Sci. USA*, **91**, 5562–5566.

- Alonso, A.D., Grundke-Iqbal, L., Barra, H.S., and Iqbal, K. (1997) Abnormal phosphorylation of tau and the mechanism of Alzheimer neurofibrillary degeneration: sequestration of microtubule-associated proteins 1 and 2 and the disassembly of microtubules by the abnormal tau. *Proc. Natl. Acad. Sci. USA*, **94**, 298–303.
- Andreadis, A., Brown, W.M., and Kosik, K.S. (1992) Structure and novel exons of the human tau gene. *Biochemistry*, **31**, 10626–10633.
- Armstrong, R.A., Winsper, S.J., and Blair, J.A. (1996) Aluminium and Alzheimer's disease: review of possible pathogenic mechanisms. *Dementia*, **7**, 1–9.
- Arnold, C.S., Johnson, G.V.W., Cole, R.N., Dong, D.L., Le, M., and Hart, G.W. (1996) The microtubule-associated protein tau is extensively modified with O-linked N-acetylglucosamine. *J. Biol. Chem.*, **271**, 28741–28744.
- Bada, J.L. (1985) Racemization of amino acids. In *Chemistry and biochemistry of the amino acids*. G.C. Barrett, editors. Chapman and Hall, New York. pp. 399–414.
- Biernat, J., Gustke, N., Drewes, G., Mandelkow, E.-M., and Mandelkow, E. (1993) Phosphorylation of Ser²⁶² strongly reduces binding of tau to microtubules: distinction between PHF-like immunoreactivity and microtubule binding. *Neuron*, **11**, 153–163.
- Binder, L.I., Frankfurter, A., and Rebhun, L.I. (1985) The distribution of tau in the mammalian central nervous system. *J. Cell Biol.*, **101**, 1371–1378.
- Black, M.M., Slaughter, T., Moshiaich, S., Obrocka, M., and Fischer, I. (1996) Tau is enriched on dynamic microtubules in the distal region of growing axons. *J. Neurosci.*, **16**, 3601–3619.
- Bondareff, W., Harrington, C., Wischik, C.M., Hauser, D.L., and Roth, M. (1994) Immunohistochemical staging of neurofibrillary degeneration in Alzheimer's disease. *J. Neuropathol. Exp. Neurol.*, **53**, 158–164.
- Braak, E., Braak, H., and Mandelkow, E.M. (1994) A sequence of cytoskeleton changes related to the formation of neurofibrillary tangles and neuropil threads. *Acta Neuropathol. Berl.*, **87**, 554–567.
- Braak, H., Braak, E., and Bohl, J. (1993) Staging of Alzheimer-related cortical destruction. *Eur. Neurol.*, **33**, 403–408.
- Brandt, R., and Lee, G. (1993a) The balance between tau protein's microtubule growth and nucleation activities: Implications for the formation of axonal microtubules. *J. Neurochem.*, **61**, 997–1005.
- Brandt, R., and Lee, G. (1993b) Functional organization of microtubule-associated protein tau. Identification of regions which affect microtubule growth, nucleation, and bundle formation *in vitro*. *J. Biol. Chem.*, **268**, 3414–3419.
- Brandt, R., and Lee, G. (1994) Orientation, assembly, and stability of microtubule bundles induced by a fragment of tau protein. *Cell Motil. Cytoskel.*, **28**, 143–154.
- Brandt, R., Lee, G., Teplow, D.B., Shalloway, D., and Abdel-Ghany, M. (1994) Differential effect of phosphorylation and substrate modulation on tau's ability to promote microtubule growth and nucleation. *J. Biol. Chem.*, **269**, 11776–11782.
- Brandt, R., Léger, J., and Lee, G. (1995) Interaction of tau with the neural plasma membrane mediated by tau's amino-terminal projection domain. *J. Cell Biol.*, **131**, 1327–1340.
- Brion, J.B., Passareiro, J.P., Nunez, J., and Flament-Durand, J. (1985) Mise en évidence immunologique de la protéine tau au niveau des lésions de dégénérescence neurofibrillaire de la maladie d'Alzheimer. *Arch. Biol. (Bruxelles)*, **95**, 229–235.
- Brion, J.P., Guilleminot, J., Couchie, D., Flament, D.J., and Nunez, J. (1988) Both adult and juvenile tau microtubule-associated proteins are axon specific in the developing and adult rat cerebellum. *Neuroscience*, **25**, 139–146.
- 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.

- Cáceres, A., and Kosik, K.S. (1990) Inhibition of neurite polarity by tau antisense oligonucleotides in primary cerebellar neurons. *Nature*, **343**, 461–463.
- Cáceres, A., Potrebic, S., and Kosik, K.S. (1991) The effect of tau antisense oligonucleotides on neurite formation of cultured cerebellar macroneurons. *J. Neuroscience*, **11**, 1515–1523.
- Chen, J., Kanai, Y., Cowan, N.J., and Hirokawa, N. (1992) Projection domains of MAP2 and tau determine spacings between microtubules in dendrites and axons. *Nature*, **360**, 674–677.
- Cleveland, D.W., Hwo, S.Y., and Kirschner, M.W. (1977a) Physical and chemical properties of purified tau factor and the role of tau in microtubule assembly. *J. Mol. Biol.*, **116**, 227–247.
- Cleveland, D.W., Hwo, S.Y., and Kirschner, M.W. (1977b) Purification of tau, a microtubule-associated protein that induces assembly of microtubules from purified tubulin. *J. Mol. Biol.*, **116**, 207–225.
- Correas, I., Diaz, N.J., and Avila, J. (1992) Microtubule-associated protein tau is phosphorylated by protein kinase C on its tubulin binding domain. *J. Biol. Chem.*, **267**, 15721–15728.
- Correas, I., Padilla, R., and Avila, J. (1990) The tubulin-binding sequence of brain microtubule-associated proteins, tau and MAP-2, is also involved in actin binding. *Biochem. J.*, **269**, 61–64.
- Cummings, B.J., Satou, T., Head, E., Milgram, N.W., Cole, G.M., Savage, M.J., Podlisny, M.B., Selkoe, D.J., Siman, R., Greenberg, B.D., and Cotman, C.W. (1996) Diffuse plaques contain C-terminal A β 42 and not A β 40: Evidence from cats and dogs. *Neurobiol. Aging*, **17**, 653–659.
- Davis, D.R., Brion, J.P., Couck, A.M., Gallo, J.M., Hanger, D.P., Ladhani, K., Lewis, C., Miller, C.C., Rupniak, T., Smith, C., and Anderton, B.H. (1995) The phosphorylation state of the microtubule-associated protein tau as affected by glutamate, colchicine and beta-amyloid in primary rat cortical neuronal cultures. *Biochem. J.*, **309**, 941–949.
- DiTella, M., Feiguin, F., Morfini, G., and Cáceres, A. (1994) Microfilament-associated growth cone component depends upon tau for its intracellular localization. *Cell Motil. Cytoskel*, **29**, 117–130.
- Drechsel, D.N. (1997) Using video microscopy to study tau's effects on the dynamic instability of microtubule assembly. In *Brain microtubule associated proteins: Modifications in disease*. J. Avila, R. Brandt, and K.S. Kosik, editors. Harwood, Amsterdam, pp. 275–292.
- Drechsel, D.N., Hyman, A.A., Cobb, M.H., and Kirschner, M.W. (1992) Modulation of the dynamic instability of tubulin assembly by the microtubule associated protein tau. *Mol. Biol. Cell*, **3**, 1141–1154.
- Drewes, G., Trinczek, B., Illenberger, S., Biernat, J., Schmitt-Ulms, G., Meyer, H.E., Mandelkow, E.M., and Mandelkow, E. (1995) Microtubule-associated protein/microtubule affinity-regulating kinase (p110mark). A novel protein kinase that regulates tau-microtubule interactions and dynamic instability by phosphorylation at the Alzheimer-specific site serine 262. *J. Biol. Chem.*, **270**, 7679–7688.
- Dudek, S.M., and Johnson, G.V. (1993) Transglutaminase catalyzes the formation of sodium dodecyl sulfate-insoluble, Alz-50-reactive polymers of tau. *J. Neurochem.*, **61**, 1159–1162.
- Dustin, P., and Flament-Durand, J. (1982) Disturbances of axoplasmic transport in Alzheimer's disease. In *Axoplasmic transport in physiology and pathology*. D.G. Weiss, and A. Gorio, editors. Springer, Heidelberg, pp. 131–136.
- Edson, K., Weisshaar, B., and Matus, A. (1993) Actin depolymerisation induces process formation on MAP2-transfected non-neuronal cells. *Development*, **117**, 689–700.
- Feany, M.B., Mattiace, L.A., and Dickson, D.W. (1996) Neuropathologic overlap of progressive supranuclear palsy, Pick's disease and corticobasal degeneration. *J. Neuropathol. Exp. Neurol.*, **55**, 53–67.
- Francon, J., Lennon, A.M., Fellous, A., Mareck, A., Pierre, M., and Nunez, J. (1982) Heterogeneity of microtubule-associated proteins and brain development. *Eur. J. Biochem.*, **129**, 465–471.

- Games, D., Adams, D., Alessandrini, R., Barbour, R., Berthelette, P., Blackwell, C., Carr, T., Clemens, 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., Montaya-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 β -amyloid precursor protein. *Nature*, **373**, 523–527.
- Garver, T.D., Lehman, R.A., and Billingsley, M.L. (1996) Microtubule assembly competence analysis of freshly-biopsied human tau, dephosphorylated tau, and Alzheimer tau. *J. Neurosci. Res.*, **44**, 12–20.
- 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.
- Goode, B.L., Denis, P.E., Panda, D., Radeke, M.J., Miller, H.R., Wilson, L., and Feinstein, S.C. (1997) Functional interactions between the proline-rich and repeat regions of tau enhance microtubule binding and assembly. *Mol Biol. Cell*, **8**, 353–365.
- Götz, J., Probst, A., Spillantini, M.G., Schäfer, T., Jakes, R., Bürki, K., and Goedert, M. (1995) Somatodendritic localization and hyperphosphorylation of tau protein in transgenic mice expressing the longest human brain tau isoform. *EMBO J.*, **14**, 1304–1313.
- Griffith, L.M., and Pollard, T.D. (1978) Evidence for actin filament-microtubule interaction mediated by microtubule-associated proteins. *J. Cell Biol.*, **78**, 958–965.
- Griffith, L.M., and Pollard, T.D. (1982) The interaction of actin with microtubules and microtubule-associated proteins. *J. Biol. Chem.*, **257**, 9143–9151.
- Grundke, I.I., Iqbal, K., Tung, Y.C., Quinlan, M., Wisniewski, H.M., and Binder, L.I. (1986) Abnormal phosphorylation of the microtubule-associated protein tau in Alzheimer cytoskeletal pathology. *Proc. Natl Acad. Sci. USA*, **83**, 4913–4917.
- Gu, Y., Oyama, F., and Ihara, Y. (1996) Tau is widely expressed in rat tissues. *J. Neurochem.*, **67**, 1235–1244.
- Gustke, N., Trinczek, B., Biernat, J., Mandelkow, E.-M., and Mandelkow, E. (1994) Domains of tau protein and interactions with microtubules. *Biochemistry*, **33**, 9511–9522.
- Guttmann, R.P., Erickson, A.C., and Johnson, G.V. (1995) Tau self-association: stabilization with a chemical cross-linker and modulation by phosphorylation and oxidation state. *J. Neurochem.*, **64**, 1209–1215.
- Hagestedt, 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.
- Harada, A., Oguchi, K., Okabe, S., Kuno, J., Terada, S., Ohshima, T., Sato-Yoshitake, R., Takei, Y., Noda, T., and Hirokawa, N. (1994) Altered microtubule organization in small-calibre axons of mice lacking tau protein. *Nature*, **369**, 488–491.
- Harrington, C.R., and Colaco, C.A.L.S. (1997) Glycation of tau protein. In *Brain microtubule associated proteins: Modifications in disease*. J. Avila, R. Brandt, and K. S. Kosik, editors. Harwood, Amsterdam, pp. 125–152.
- Hayes, B.K., and Hart, G.W. (1994) Novel forms of protein glycosylation. *Curr. Opin. Struct. Biol.*, **4**, 692–696.
- Heilbroner, L.P., and Kemper, T.L. (1990) The cytoarchitectonic distribution of senile plaques in three aged monkeys. *Acta Neuropathol.*, **81**, 60–65.
- Hershko, A., and Ciechanover, A. (1992) The ubiquitin system for protein degradation. *Annu. Rev. Biochem.*, **61**, 761–807.

- Higgins, L.S., Rodems, J.M., Catalano, R., Quon, D., and Cordell, B. (1995) Early Alzheimer disease-like histopathology increases in frequency with age in mice transgenic for β -APP751. *Proc. Natl Acad. Sci. USA*, **92**, 4402–4406.
- Hirokawa, N., Funakoshi, T., Satoharada, R., and Kanai, Y. (1996) Selective stabilization of tau in axons and microtubule associated protein 2c in cell bodies and dendrites contributes to polarized localization of cytoskeletal proteins in mature neurons. *J. Cell Biol.*, **132**, 667–679.
- Hong, M., and Lee, V.M.Y. (1997) Insulin and insulin like growth factor 1 regulate tau phosphorylation in cultured human neurons. *J. Biol Chem.*, **272**, 19547–19553.
- Hoshi, M., Takashima, A., Noguchi, K., Murayama, M., Sato, M., Kondo, S., Saitoh, Y., Ishiguro, K., Hoshino, T., and Imahori, K. (1996) Regulation of mitochondrial pyruvate dehydrogenase activity by tau protein kinase I/glycogen synthase kinase 3 β in brain. *Proc. Natl Acad. Sci. USA*, **93**, 2719–2723.
- 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.
- Huang, D.Y., Weisgraber, K.H., Goedert, M., Saunders, A.M., Roses, A.D., and Strittmatter, W.J. (1995) ApoE3 binding to tau tandem repeat I is abolished by tau serine262 phosphorylation. *Neurosci. Lett.*, **192**, 209–212.
- Iqbal, K., Zaidi, T., Bancher, C., and Grundke-Iqbal, I. (1994) Alzheimer paired helical filaments. Restoration of the biological activity by dephosphorylation. *FEBS Lett.*, **349**, 104–108.
- Ishiguro, K., Omori, A., Takamatsu, M., Sato, K., Arioka, M., Uchida, T., and Imahori, K. (1992) Phosphorylation sites on tau by tau protein kinase I, a bovine derived kinase generating an epitope of paired helical filaments. *Neurosci. Lett.*, **148**, 202–206.
- Ishiguro, K., Shiratsuchi, A., Sato, S., Omori, A., Arioka, M., Kobayashi, S., Uchida, T., and Imahori, K. (1993) Glycogen synthase kinase 3 beta is identical to tau protein kinase I generating several epitopes of paired helical filaments. *FEBS Lett.*, **325**, 167–172.
- Kaech, S., Ludin, B., and Matus, A. (1996) Cytoskeletal plasticity in cells expressing neuronal microtubule-associated proteins. *Neuron*, **17**, 1189–1199.
- Kampers, T., Fridhoff, P., Biernat, J., Mandelkow, E.M., and Mandelkow, E. (1996) RNA stimulates aggregation of microtubule-associated protein tau into Alzheimer-like paired helical filaments. *FEBS Lett.*, **399**, 344–349.
- Kanai, Y., and Hirokawa, N. (1995) Sorting mechanisms of tau and MAP2 in neurons: suppressed axonal transit of MAP2 and locally regulated microtubule binding. *Neuron*, **14**, 421–432.
- Kawahara, M., Muramoto, K., Kobayashi, K., and Kuroda, Y. (1992) Functional and morphological changes in cultured neurons of rat cerebral cortex induced by long-term application of aluminium. *Biochem. Biophys. Res. Commun.*, **189**, 1317–1322.
- Kayyali, U.S., Zhang, W., Yee, A.G., Seidman, J.G., and Potter, H. (1997) Cytoskeletal changes in the brains of mice lacking calcineurin A α . *J. Neurochem.*, **68**, 1668–1678.
- Kempf, M., Clement, A., Faissner, A., Lee, G., and Brandt, R. (1996) Tau binds to the distal axon early in development of polarity in a microtubule- and microfilament-dependent manner. *J. Neurosci.*, **16**, 5583–5592.
- Kenessey, A., and Yen, S.H. (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.
- Kenessey, A., Yen, S.H., Liu, W.K., Yang, X.R., and Dunlop, D.S. (1995) Detection of D-aspartate in tau proteins associated with Alzheimer paired helical filaments. *Brain Res.*, **675**, 183–189.
- Kenner, L., el-Shabrawi, Y., Hutter, H., Forstner, M., Zatloukal, K., Hoefler, G., Preisegger, K.H., Kurzbauer, R., and Denk, H. (1994) Expression of three- and four-repeat tau isoforms in mouse liver. *Hepatology*, **20**, 1086–1089.

- Kimura, T., Ono, T., Takamatsu, J., Yamamoto, H., Ikegami, K., Kondo, A., Hasegawa, M., Ihara, Y., Miyamoto, E., and Miyakawa, T. (1996) Sequential changes of tau-site-specific phosphorylation during development of paired helical filaments. *Dementia*, **7**, 177–181.
- Knops, J., Kosik, K.S., Lee, G., Pardee, J.D., Cohen, G.L., and McConlogue, L. (1991) Overexpression of tau in a nonneuronal cell induces long cellular processes. *J. Cell Biol.*, **114**, 725–733.
- Kondo, J., Honda, T., Mon, H., Hamada, Y., Miura, R., Ogawara, M., and Ihara, Y. (1988) The carboxyl third of tau is tightly bound to paired helical filaments. *Neuron*, **1**, 827–834.
- Kosik, K.S., Orecchio, L.D., Bakalis, S., and Neve, R.L. (1989) Developmentally regulated expression of specific tau sequences. *Neuron*, **2**, 1389–1397.
- Ksiezak-Reding, H., Liu, W.K., Wall, J.S., and Yen, S.H. (1997) Biochemical isolation and characterization of paired helical filaments. In *Brain microtubule associated proteins: Modifications in disease*. J. Avila, R. Brandt, and K. Kosik, editors. Harwood, Amsterdam, pp. 331–362.
- Ksiezak-Reding, H., Shafit-Zagardo, B., and Yen, S.H. (1995) Differential expression of exons 10 and 11 in normal tau and tau associated with paired helical filaments. *J. Neurosci. Res.*, **41**, 583–593.
- Ksiezak-Reding, H., Tracz, E., Yang, L.S., Dickson, D.W., Simon, M., and Wall, J.S. (1996) Untrastructural instability of paired helical filaments from corticobasal degeneration as examined by scanning transmission electron microscopy. *Am. J. Pathol.*, **149**, 639–652.
- Lambert, M.P., Stevens, G., Sabo, S., Barber, K., Wang, G., Wade, W., Krafft, G., Snyder, S., 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.
- 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., Colaco, P., and Avila, J. (1995) Tau protein from Alzheimer's disease patients is glycosylated at its tubulin-binding domain. *J. Neurochem.*, **65**, 1658–1664.
- Ledesma, M.D., Medina, M., and Avila, J. (1996) The in vitro formation of recombinant tau polymers: effect of phosphorylation and glycation. *Mol. Chem. Neuropathol.*, **27**, 249–258.
- Lee, G. (1997) Expression of MAP cDNAs in eukaryotic cells. In *Brain microtubule associated proteins: Modifications in disease*. J. Avila, R. Brandt, and K. S. Kosik, editors. Harwood, Amsterdam, pp. 293–311.
- Lee, G., Neve, R.L., and Kosik, K.S. (1989) The microtubule binding domain of tau protein. *Neuron*, **2**, 1615–1624.
- Léger, J., Brandt, R., and Lee, G. (1994) Identification of tau protein regions required for process formation in PC12 cells. *J. Cell Sci.*, **107**, 3403–3412.
- Léger, J., Kempf, M., Lee, G., and Brandt, R. (1997) Conversion of serine to aspartate imitates phosphorylation-induced changes in the structure and function of microtubule-associated protein tau. *J. Biol. Chem.*, **272**, 8441–8446.
- Litersky, J.M., and Johnson, G.V. (1992) Phosphorylation by cAMP-dependent protein kinase inhibits the degradation of tau by calpain. *J. Biol. Chem.*, **267**, 1563–1568.
- Litman, P., Barg, J., Rindzoonski, L., and Ginzburg, I. (1993) Subcellular localization of tau mRNA in differentiating neuronal cell culture: implications for neuronal polarity. *Neuron*, **10**, 627–638.
- Liu, W.K., Dickson, D.W., and Yen, S.H. (1993a) Heterogeneity of tau protein in Alzheimer's disease. Evidence for increased expression of an isoform and preferential distribution of a phosphorylated isoform in neurites. *Am. J. Pathol.*, **142**, 387–394.
- Liu, W.K., Ksiezak-Reding, H., and Yen, S.H. (1991) Abnormal proteins from Alzheimer's disease brain. Purification and amino acid analysis. *J. Biol. Chem.*, **266**, 21723–21727.

- Liu, W.K., Moore, W.T., Williams, R.T., Hall, F.L., and Yen, S.H. (1993b) Application of synthetic phospho- and unphospho-peptides to identify phosphorylation sites in a subregion of the tau molecule, which is modified in Alzheimer's disease. *J. Neurosci. Res.*, **34**, 371–376.
- Liu, W.K., and Yen, S.H. (1996) The state of phosphorylation of normal adult brain tau, fetal tau, and tau from Alzheimer paired helical filaments at amino acid residue Ser262. *J. Neurochem.*, **66**, 1131–1139.
- Lowe, J., Mayer, R.J., and Landon, M. (1993) Ubiquitin in neurodegenerative diseases. *Brain Pathol.*, **3**, 55–65.
- Lu, Q., and Wood, J.G. (1993) Functional studies of Alzheimer's disease tau proteins. *J. Neurosci.*, **13**, 508–515.
- 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.
- Mandelkow, E., Song, Y.H., Schweers, O., Marx, A., and Mandelkow, E.M. (1995) On the structure of microtubules, tau, and paired helical filaments. *Neurobiol. Aging*, **16**, 347–354.
- Mandell, J.W., and Banker, G.A. (1996) A spatial gradient of tau protein phosphorylation in nascent axons. *J. Neurosci.*, **16**, 5727–5740.
- Martin, H., Lambert, M.P., Barber, K., Hinton, S., and Klein, W.L. (1995) Alzheimer's-associated phospho-epitope in human neuroblastoma cell cultures: up-regulation by fibronectin and laminin. *Neuroscience*, **66**, 769–779.
- Martin, L.J., Sisodia, S.S., Koo, E.H., Cork, L.C., Dellovade, T.L., Weidemann, A., Beyreuther, K., Masters, C., and Price, D.L. (1991) Amyloid precursor protein in aged nonhuman primates. *Proc. Natl Acad. Sci. USA*, **88**, 1461–1465.
- Matsuo, E.S., Shin, R., Billingsley, M.L., deVoorde, A.V., O'Connor, M., Trojanowski, J.Q., and Lee, V.M. (1994) Biopsy-derived adult human brain tau is phosphorylated at many of the same sites as Alzheimer's disease paired helical filament tau. *Neuron*, **13**, 989–1002.
- Mattson, M.P., Cheng, B., Davis, D., Bryant, K., Lieberburg, I., and Rydel, R.E. (1992) Beta-amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *J. Neurosci.*, **12**, 376–389.
- Mattson, M.P., Lovell, M.A., Ehmman, W.D., and Markesbery, W.R. (1993) Comparison of the effects of elevated intracellular aluminium and calcium levels on neuronal survival and tau immunoreactivity. *Brain Res.*, **602**, 21–31.
- Mercken, M., Grynspan, F., and Nixon, R.A. (1995) Differential sensitivity proteolysis by brain calpain of adult human tau, fetal human tau and PHF-tau. *FEBS Lett.*, **368**, 10–14.
- Mori, H. (1997) Ubiquitination of tau as an abnormal protein. In *Brain microtubule associated proteins: Modifications in disease*. J.Avila, R.Brandt, and K.S.Kosik, editors. Harwood, Amsterdam, pp. 113–124.
- Mori, H., Kondo, J., and Ihara, Y. (1987) Ubiquitin is a component of paired helical filaments in Alzheimer's disease. *Science*, **235**, 1641–1644.
- Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Titani, K., and Ihara, Y. (1993) Ubiquitin is conjugated with amino-terminally processed tau in paired helical filaments. *Neuron*, **10**, 1151–1160.
- Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Yoshida, H., Titani, K., and Ihara, Y. (1995) Proline-directed and non-proline-directed phosphorylation of PHF-tau. *J. Biol. Chem.*, **270**, 823–829.
- Murphy, D.B., Johnson, K.A., and Borisy, G.G. (1977) Role of tubulin-associated proteins in microtubule nucleation and elongation. *J. Mol. Biol.*, **117**, 33–52.
- Nakamura, S., Kiatipattanasakul, W., Nakayama, H., Ono, F., Sakakibara, I., Yoshikawa, Y., Goto, N., and Doi, K. (1996) Immunohistochemical characteristics of the constituents of senile plaques and amyloid angiopathy in aged cynomolgus monkeys. *J. Med. Primatol.*, **25**, 294–300.

- Nelson, P.T., and Saper, C.B. (1995) Ultrastructure of neurofibrillary tangles in the cerebral cortex of sheep. *Neurobiol Aging*, **16**, 315–323.
- Papasozomenos, S.C., and Binder, L.I. (1987) Phosphorylation determines two distinct species of tau in the central nervous system. *Cell Motil Cytoskel*, **8**, 210–226.
- Paudel, H.K. (1997) The regulatory Ser262 of microtubule-associated protein tau is phosphorylated by phosphorylase kinase. *J. Biol Chem.*, **272**, 1777–1785.
- Pleasure, S.J., Page, C., and Lee, V.M.Y. (1992) Pure, postmitotic, polarized human neurons derived from NTera 2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. *J. Neurosci.*, **12**, 1802–1815.
- Pollock, N.J., Mirra, S.S., Binder, L.I., Hansen, L.A., and Wood, J.G. (1986) Filamentous aggregates in Pick's disease, progressive supranuclear palsy, and Alzheimer's disease show antigenic de-terminants with microtubule-associated protein tau. *Lancet*, **2**, 1211.
- Roses, A.D., Einstein, G., Gilbert, J., Goedert, M., Han, S.H., Huang, D., Hulette, C., Masliah, E., Pericak-Vance, M.A., Saunders, A.M., Schmechel, D.E., Strittmatter, W.J., Weisgraber, K.H., and Xi, P.T. (1996) Morphological, biochemical, and genetic support for an apolipoprotein E effect on microtubular metabolism. *Ann. N.Y. Acad. Sci.*, **777**, 146–157.
- Sautiere, P.E., Sindou, P., Couratier, P., Hugon, J., Watzet, A., and Delacourte, A. (1992) Tau antigenic changes induced by glutamate in rat primary culture model: a biochemical approach. *Neurosci. Lett.*, **140**, 206–210.
- 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.
- 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.
- Scott, C.W., Fieles, A., Sygowski, L.A., and Caputo, C.B. (1993a) Aggregation of tau protein by aluminium. *Brain Res.*, **628**, 77–84.
- Scott, C.W., Spreen, R.C., Herman, J.L., Chow, F.P., Davison, M.D., Young, J., and Caputo, C.B. (1993b) Phosphorylation of recombinant tau by cAMP-dependent protein kinase. Identification of phosphorylation sites and effect on microtubule assembly. *J. Biol Chem.*, **268**, 1166–1173.
- Selden, C.S., and Pollard, T.D. (1983) Phosphorylation of microtubule-associated proteins regulates their interaction with actin filaments. *J. Biol Chem.*, **258**, 7064–7071.
- Seubert, P., Mawal-Dewan, M., Barbour, R., Jakes, R., Goedert, M., Johnson, G.V.W., Litersky, J.M., Schenk, D., Lieberburg, I., Trojanowski, J.Q., and Lee, V.M.Y. (1995) Detection of phosphorylated Ser262 in fetal tau, adult tau, and paired helical filament tau. *J. Biol Chem.*, **270**, 18917–18922.
- Sindou, P., Couratier, P., Barthe, D., and Hugon, J. (1992) A dose-dependent increase of tau immunostaining is produced by glutamate toxicity in primary neuronal cultures. *Brain Res.*, **572**, 242–246.
- Sindou, P., Lesort, M., Couratier, P., Yardin, C., Esclaire, F., and Hugon, J. (1994) Glutamate increases tau phosphorylation in primary neuronal cultures from fetal rat cerebral cortex. *Brain Res.*, **646**, 124–128.
- Smith, M.A., Taneda, S., Richey, P.L., Miyata, S., Yan, S.D., Stern, D., Sayre, L.M., Monnier, V.M., and Perry, G. (1994) Advanced Maillard reaction end products are associated with Alzheimer's disease pathology. *Proc. Natl Acad. Sci. USA*, **91**, 5710–5714.
- Steiner, B., Mandelkow, E.M., Biernat, J., Gustke, N., Meyer, H.E., Schmidt, B., Mieskes, G., Soling, H.D., Drechsel, D., Kirschner, M.W., Goedert, M., and Mandelkow, E. (1990) Phosphorylation of microtubule-associated protein

- tau: identification of the site for Ca²⁺-calmodulin dependent kinase and relationship with tau phosphorylation in Alzheimer tangles. *EMBO J.*, **9**, 3539–3544.
- Strittmatter, W.J., and Roses, A.D. (1995) Apolipoprotein E and Alzheimer disease. *Proc. Natl. Acad. Sci. USA*, **92**, 4725–4727.
- Strittmatter, W.J., Saunders, A.M., Goedert, M., Weisgraber, K.H., Dong, L.M., Jakes, R., Huang, D.Y., Pericak-Vance, M., Schmechel, D., and Roses, A.D. (1994) Isoform-specific interactions of apolipoprotein E with microtubule-associated protein tau: implications for Alzheimer disease. *Proc. Natl. Acad. Sci. USA*, **91**, 11183–11186.
- Szendrei, G.L., Lee, V.M., and Otvos, L.J. (1993) Recognition of the minimal epitope of monoclonal antibody Tau-1 depends upon the presence of a phosphate group but not its location. *J. Neurosci. Res.*, **34**, 243–249.
- Takashima, A., Noguchi, K., Michel, G., Mercken, M., Hoshi, M., Ishiguro, K., and Imahori, K. (1996) Exposure of rat hippocampal neurons to amyloid beta peptide (25–35) induces the inactivation of phosphatidylinositol-3 kinase and the activation of tau protein kinase I/glycogen synthase kinase-3 beta. *Neurosci. Lett.*, **203**, 33–36.
- Takashima, A., Noguchi, K., Sato, K., Hishino, T., and Imahori, K. (1993) Tau protein kinase I is essential for amyloid beta-protein-induced neurotoxicity. *Proc. Natl. Acad. Sci. USA*, **90**, 7789–7793.
- Tekirian, T.L., Cole, G.M., Russell, M.J., Wekstein, D.R., Patel, E., Snowden, D.R., Markesbery, W.R., and Geddes, J.W. (1996) Carboxy terminal of β -amyloid deposits in aged human, canine and polar bear brains. *Neurobiol. Aging*, **17**, 249–257.
- Trinczek, B., Biernat, J., Baumann, K., Mandelkow, E.M., and Mandelkow, E. (1995) Domains of tau protein, differential phosphorylation, and dynamic instability of microtubules. *Mol. Biol. Cell*, **6**, 1887–1902.
- Trojanowski, J.Q., Schuck, T., Schmidt, M.L., and Lee, V.M. (1989) Distribution of tau proteins in the normal human central and peripheral nervous system. *J. Histochem. Cytochem.*, **37**, 209–215.
- Vincent, I., Rosado, M., Kim, E., and Davies, P. (1994) Increased production of paired helical filament epitopes in a cell culture system reduces the turnover of tau. *J. Neurochem.*, **62**, 715–723.
- Vulliamy, R., Halloran, M., Smith, A., and Lee, G. (1992) Proline-directed phosphorylation of human tau protein. *J. Biol. Chem.*, **267**, 22570–22574.
- Wang, J.Z., Grundke-Iqbal, I., and Iqbal, K. (1996a) Glycosylation of microtubule-associated protein tau: an abnormal posttranslational modification in Alzheimer's disease. *Nature Med.*, **2**, 871–875.
- Wang, J.Z., Grundke-Iqbal, I., and Iqbal, K. (1996b) Restoration of biological activity of Alzheimer abnormally phosphorylated tau by dephosphorylation with protein phosphatase-2A,-2B and 1. *Brain Res. Mol. Brain Res.*, **38**, 200–208.
- Watanabe, A., Hasegawa, M., Suzuki, M., Takio, K., Morishima-Kawashima, M., Titani, K., Arai, T., Kosik, K.S., and Ihara, Y. (1993) *In vivo* phosphorylation sites in fetal and adult rat tau. *J. Biol. Chem.*, **268**, 25712–25717.
- Weingarten, M.D., Lockwood, A.H., Hwo, S.Y., and Kirschner, M.W. (1975) A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. USA*, **72**, 1858–1862.
- Wischik, C.M., Edwards, P.C., Lai, R.Y.K., Roth, M., and Harrington, C.R. (1996) Selective inhibition of Alzheimer disease-like tau aggregation by phenothiazines. *Proc. Natl. Acad. Sci. USA*, **93**, 11213–11218.
- Yan, S.D., Chen, X., Schmidt, A.M., Brett, J., Godman, G., Zou, Y.S., C.W., S., Caputo, C., Frappier, T., Smith, M.A., Perry, G., Yen, S.H., and Stern, D. (1994) Glycated tau protein in Alzheimer disease: A mechanism for induction of oxidant stress. *Proc. Natl. Acad. Sci. USA*, **91**, 7787–7791.
- Yang, L.S., and Ksiazek-Reding, H. (1995) Association with paired helical filaments protects tau from calpain-induced proteolysis. *J. Neurochem.*, **64**, S54B.

- Yang, S.D., Song, J.S., Yu, J.S., and Shiah, S.G. (1993) Protein kinase FA/GSK-3 phosphorylates tau on Ser235-Pro and Ser404-Pro that are abnormally phosphorylated in Alzheimer's disease brain. *J. Neurochem.*, **61**, 1742–1747.
- Yang, S.D., Yu, J.S., Shiah, S.G., and Huang, J.J. (1994) Protein kinase FA/glycogen synthase kinase3 a after heparin potentiation phosphorylates tau on sites abnormally phosphorylated in Alzheimer's disease brain. *J. Neurochem.*, **63**, 1416–1425.
- Yankner, B.A., Duffy, L.K., and Kirschner, D.A. (1990) Neurotrophic and neurotoxic effect of amyloid β protein: reversal by tachykinin neuropeptides. *Science*, **250**, 279–282.
- Yoshida, H., and Ihara, Y. (1993) Tau in paired helical filaments is functionally distinct from fetal tau: assembly incompetence of paired helical filament-tau. *J. Neurochem.*, **61**, 1183–1186.
- Zatloukal, K., Kenner, L., Preisegger, K.H., and Denk, H. (1995) Alcoholic liver disease: molecularpathologic aspects. *Verh. Dtsch. Ges. Pathol.*, 28–35.

THE GENETICS OF FAMILIAL ALZHEIMER'S DISEASE

3.

MUTATIONS IN THREE GENES ARE ASSOCIATED WITH EARLY ONSET ALZHEIMER'S DISEASE

PAUL E.FRASER and PETER H.ST GEORGE-HYSLOP

Centre for Research in Neurodegenerative Disease, Departments of Medical Biophysics and Medicine, University of Toronto, Department of Medicine (Neurology), The Toronto Hospital, 6 Queen's Park Crescent West, Toronto, Ontario, Canada M5S 3H2

GENETIC EPIDEMIOLOGY OF ALZHEIMER'S DISEASE

Familial aggregation of Alzheimer's disease (AD) has been recognized since the earliest formal descriptions of this disease. The degree to which genetic risk factors play a role in the pathogenesis of AD however has been less clear. In the past several years, a number of genetic epidemiology studies have been undertaken on probands with AD and their families (Heyman *et al.*, 1984; Rocca *et al.*, 1986; Breitner *et al.*, 1988; Farrer *et al.*, 1991; Bergem *et al.*, 1992; Katzman and Kawas, 1994; Lautenschlager *et al.*, 1996). Cumulatively, these studies (for review see, Katzman and Kawas, 1994) strongly argue that the familial aggregation of AD is not due simply to the high frequency of AD in the general population. These studies suggest that the age-dependent risk and the overall lifetime risk for AD in first-degree relatives of AD probands varies from 10–50%. The most comprehensive recent study suggests an age-dependent risk curve asymptotic to a final risk of 38% by age 85 years (Lautenschlager *et al.*, 1996). The latter study, as well as several other earlier epidemiologic studies make it difficult to assign a pure Mendelian mode of transmission in the majority of AD cases. Instead, these studies and also studies of individual pedigrees imply that the majority of cases of familially aggregated AD probably reflect a complex mode of transmission such as a common but incompletely-penetrant single autosomal gene defect, a multi-genic trait, or more probably a mode of transmission in which genetic and environmental factors interact. Nevertheless, there is a small proportion of AD cases which appear to be transmitted as a pure autosomal dominant Mendelian trait with age-dependent penetrance (for review see, St George-Hyslop *et al.*, 1987). Analysis of these pedigrees with molecular genetic tools has provided several powerful insights into the pathogenesis of AD.

MOLECULAR GENETICS OF ALZHEIMER'S DISEASE

Molecular genetic studies in pedigrees with a reasonably unambiguous single gene auto-somal dominant pattern of inheritance have led to the identification of the four different genes associated with

inherited susceptibility to AD. Because a significant number of pedigrees multiply affected by AD have been found which do not segregate mutations/ polymorphisms in any of the four known AD susceptibility genes, it is suspected that at least one and possibly several additional AD susceptibility genes must also exist but have not yet been identified (Sherrington *et al.*, 1996).

There are two common strategies which are used to isolate disease genes. The most straight-forward of the two strategies is to clone a "candidate gene" whose role in the disease has been suggested on the basis of its biochemical function in relation to known biochemical phenotypes associated with the disease. This method obviously requires a significant knowledge of the biochemical pathophysiology of the disease in order that the search for disease related mutations or polymorphisms can be focused upon a few likely candidate genes. For most neurodegenerative diseases like Huntington's disease, spinal cerebellar ataxias, etc., this strategy has not been productive because of an inadequate knowledge of the disease biochemistry.

The other strategy typically used to identify genes causing genetic disease is based upon attempts first to define the chromosomal location of the disease gene, and then to isolate it from that delimited chromosomal location. The first step in this "positional cloning strategy" is to identify the chromosomal location by genetic linkage studies. Genetic linkage studies, employing polymorphic chromosomal markers, attempt to show co-segregation of the disease trait with a group of markers whose chromosomal location and relative order have already been defined (Gusella, 1986). Such co-segregation implies physical association between the chromosomal segment carrying the disease gene and the chromosomal segments carrying the markers. Once the approximate chromosomal location has been defined, it may become immediately possible to identify potential candidate genes which have been previously mapped to that chromosomal region. Very often, however, the chromosomal region, defined by genetic linkage studies, either contains no known genes, or the genes known to map within that region fail to show biochemical or mutational evidence for involvement in the disease process. Under these conditions, additional novel candidate genes can be isolated from the genomic DNA within the linked chromosomal region (Collins, 1992). Two different methods have been devised to isolate such novel candidate genes using genomic DNA as a template. The first method, known as "exon trapping", involves cloning genomic DNA fragments into a vector which induces transcription and splicing at splice donor and acceptor sites within the genomic template sequence (Duyk *et al.*, 1990; Nisson *et al.*, 1993; Church *et al.*, 1994; Valdes *et al.*, 1994). The result is a series of putative exonic sequences which can be isolated, sequenced, and then used to recover longer full-length transcripts. The second method, known as "gene tracking", involves the use of immobilized denatured genomic DNA as a hybridization target for pooled single strand cDNA made from mRNA extracted from a tissue of interest (Tagle and Collins, 1992; Rommens *et al.*, 1993). Each method has advantages and disadvantages which are discussed in a number of reviews (see Collins, 1992).

Ultimately, whether the candidate gene is isolated on the basis of its biochemical function or is isolated through a positional cloning strategy, it is necessary to show the presence of a nucleotide sequence change which differentiates normal from affected subjects. Such nucleotide sequence changes may be deletions, amplifications, rearrangements, or simply nucleotide substitutions. The latter may exert their biochemical effect by altering the encoded amino acids of a protein, by altering consensus sequences for RNA splicing or may result in premature protein truncation by the creation of a nonsense mutation. While the demonstration of a nonsense mutation or the appearance of a structural

rearrangement within a gene provides quite good evidence that this sequence difference is pathogenic, the discrimination between a pathogenic and non-pathogenic role for subtle mutations which only alter amino acid sequence is sometimes difficult. The cumulative observations that: (1) a nucleotide missense mutation co-segregates with the disease; that the mutant sequence is never seen in a large number of normal subjects; and (2) that the mutation alters amino acid residues which are conserved in evolution are usually considered sufficient proof that the DNA sequence change is a disease-causing pathogenic mutation rather than an innocent polymorphism.

This strategy works very well for diseases with a singular phenotype, virtually complete penetrance, and either no etiologic heterogeneity or limited etiologic heterogeneity. Thus, these strategies have all been successfully applied to the analysis of the simple autosomal dominant forms of AD. For instance, the candidate gene approach led to the identification of mutations in the β APP and presenilin 2 genes. A mixture of candidate gene approach and positional cloning strategy led to the discovery of the ApoE gene, and the pure positional cloning method led to the isolation of the presenilin 1 gene.

The isolation of genes associated with complex traits like the non-autosomal dominant forms of FAD, is more difficult and is currently still in its infancy. Under circumstances of multiple disease genes (etiologic heterogeneity) or complex modes of inheritance (poly-genic inheritance or complex gene:environment interactions) the initial discovery of the chromosomal locations of the disease susceptibility loci is more difficult, and the subsequent discrimination between an innocent polymorphism and a disease-causing mutation in a candidate gene at the linked chromosomal locus may be problematic. These difficulties arise from the fact that in complex traits, there may be more than one genetic locus with additive or synergistic effects which are required to produce the disease phenotype. As a result, there may be normal subjects who carry a defective allele at one locus but not at the other loci, and therefore do not express the disease phenotype. If the other loci are not yet defined, interpretation of the significance of "variant" alleles at the test locus is difficult. The statistical methods for analysis of this type of data is currently being applied to diseases like hypertension, diabetes mellitus, and the significant proportion of the AD pedigrees where the disease is transmitted in a non-Mendelian fashion.

The Amyloid Precursor Protein

The first gene to be identified in association with inherited susceptibility to AD was the amyloid precursor protein gene (β APP). The β APP gene encodes an alternatively spliced transcript which, in its longest isoform, encodes a single transmembrane spanning polypeptide of 770 amino acids (Goldgaber *et al.*, 1987; Kang, 1987; Tanzi, 1987; Robakis *et al.*, 1988). Alternative splicing of exon 7, which encodes a Kunitz protease inhibitor protein domain and exon 8, which encodes a sequence homologous to the ox-2 antigen, result in polypeptides of 695 amino acids (which is expressed predominantly in brain), and 751 amino acids (Kitaguchi *et al.*, 1988). There have been considerable advances in the understanding of the processing of the β APP protein which are reviewed in detail elsewhere (Selkoe, 1994). The β APP precursor protein undergoes a series of endoproteolytic cleavages. One of these, which results from the action of a putative membrane-associated α -secretase, liberates the extracellular N-terminus of β APP (which was previously identified at protease nexin II) by cleavage within the A β peptide domain and is thus a non-amyloidogenic pathway because this cleavage

precludes the formation of A β peptide. The other cleavage pathway, which occurs in part in the endosomal-lysosomal compartment, involves the putative β - and δ -secretases which give rise to a series of peptides which contain the 40–42 amino acid A β peptide. A β peptides ending at residue 42 or 43 (long tailed A β) are considered to be more fibrillogenic and more neurotoxic than A β ending at residue 40 which is the predominant isoform produced during normal metabolism of β APP (Yankner *et al.*, 1990; Jarrett and Lansbury, 1993; Pike *et al.*, 1993; Lorenzo and Yanker, 1994). The activity of these enzymes, and especially δ -secretase giving rise to the more fibrillogenic and potentially neurotoxic long-tailed A β_{1-42} appear to play a central role in the pathogenesis of AD in both genetic and non-genetic forms (for reviews see, Selkoe, 1994; Yankner, 1996).

The function of bAPP is currently unknown. Knockout of the murine β APP gene is not very illuminating because it leads only to subtle phenotypes including minor weight loss, decreased locomotor activity and abnormal forelimb motor activity, and minor non-specific degrees of reactive gliosis in the cortex (Zheng *et al.*, 1996). *In vitro* studies in cultured cells suggest that secreted β APP (β APPs) can function as an autocrine factor stimulating cell proliferation, cell adhesion, and supports NGF induced neurite outgrowth of PC12 cells (Saitoh *et al.*, 1989; Milward *et al.*, 1992). Other studies have implied a role for APP in signal transduction by association of β APP with heterotrimeric GTP-binding proteins (Nishimoto *et al.*, 1993).

Several lines of evidence led to the suspicion that the β APP gene was the site of mutations associated with AD. First, patients with Down's syndrome (Trisomy 21) almost invariably develop the neuropathological attributes of AD by age 40 years (there is considerable variation in the age of onset of actual dementia which may in part be modulated by the genotype at ApoE—see below) (Wisniewski *et al.*, 1985; Wisniewski and Rabe, 1986; Robakis *et al.*, 1988; Rumble *et al.*, 1989; Hyman *et al.*, 1994; Royston *et al.*, 1996; Schupf *et al.*, 1997). Second, the gene encoding the full-length β APP protein is located on chromosome 21 (Kang *et al.*, 1987). Third, genetic linkage studies had shown weak but suggestive evidence for linkage of a familial AD locus on chromosome 21 near the markers D21S1/D21S11, which map near the β APP gene (Goate *et al.*, 1989; St George-Hyslop *et al.*, 1991). Fourth, genetic linkage and mutational studies of the β APP gene identified a Glu693Gln missense mutation of the β APP gene (codon numbering of the β APP₇₇₀ isoform) in affected and at-risk members of families with hereditary cerebral haemorrhage with amyloidosis of the Dutch type (HCHWA-D) (Levy *et al.*, 1990). Subsequently, direct nucleotide sequencing led to the discovery of several different missense mutations in exons 16 and 17 of the β APP gene in families with early-onset AD (Table 1). Some of these missense mutations are probably not pathogenic mutations either because they have also been detected also in normal elderly relatives or because they are not present in all affected members of these pedigrees. Nevertheless, the missense mutations at codon 670/671 (Swedish mutation, Mullan *et al.*, 1992), at codon 692 (Flemish mutation, Hendricks *et al.*, 1992), at codon 716, and at codon 717 (Chartier-Harlin *et al.*, 1991; Goate *et al.*, 1991; Murrell *et al.*, 1991; Naruse *et al.*, 1991; Karlinsky *et al.*, 1992) seem quite clearly to be pathogenic. The mutations at codon 670/671 and at codon 692 are rare, having been seen only in single families. Mutations at codon 717 on the other hand have been seen in several unrelated pedigrees (less than 20 pedigrees worldwide) from different ethnic origins. It may be significant, however, that the majority of such mutations at codon 717 have been seen in Anglo-Saxon, Italian and Japanese subjects. The reason for this aggregation is unclear because within each ethnic group there is no common haplotype of genetic markers within or

Table 1. Missense mutations in the β APP gene

<i>Codon</i>	<i>Mutation</i>	<i>Phenotype</i>
665	Gln->Asp	Late onset AD - no segregation (Peacock <i>et al.</i> , 1994)
670/671	Lys-Met->Asn-Leu	FAD; increased A β production (Mullan <i>et al.</i> , 1992)
673	Ala->Thr	No disease phenotype (Peacock <i>et al.</i> , 1992)
692	Ala->Gly	FAD + cerebral hemorrhage; increased A β (Hendricks <i>et al.</i> , 1992)
693	Glu->Gly	Late onset AD, no segregation (Kamino <i>et al.</i> , 1992)
	Glu->Gln	HCHWA-D (Levy <i>et al.</i> , 1990)
713	Ala->Val	Schizophrenia - no segregation (Jones <i>et al.</i> , 1992)
	Ala->Thr	AD - no segregation (Carter <i>et al.</i> , 1992)
716	Ile->Val	FAD (J. Hardy personal communication)
717	Val->Ile	FAD; increased long A β isoforms (Goate <i>et al.</i> , 1991; Naruse <i>et al.</i> , 1991; Karlinsky <i>et al.</i> , 1992)
	Val->Phe	FAD (Murrell <i>et al.</i> , 1991)
	Val->Gly	FAD (Chartier-Harlin <i>et al.</i> , 1991)

Mutations in the β APP gene. Several of the mutations (denoted—no segregation) were either observed in single affected subjects or were observed in some but not all affected family members, or were observed in affected and elderly unaffected family members. The authenticity of these mutations as disease causing mutations remains undecided.

surrounding the β APP gene (The French Alzheimer's Disease Study Group, 1996). The absence of a conserved haplotype around the β APP gene in β APP₇₁₇ carriers in the same ethnic group argues against a common founder for the β APP₇₁₇ mutation in each ethnic group.

The mechanism by which selected β APP mutations cause AD is unclear. The most simple explanation is that missense mutations in the β APP gene result in the over-production of the A β peptide and in particular, overproduction of long-tailed isoforms ending at residue 42 or 43 (Cai *et al.*, 1992; Citron *et al.*, 1992; Haass *et al.*, 1994; Susuki *et al.*, 1994; Haass *et al.*, 1995). Studies from a variety of groups suggest that A β , like several other amyloids, exhibits neurotoxicity when aggregated as a fibril, and imply that a conformational change is necessary to change the inert (or even marginally neurotrophic) soluble A β into toxic A β (Yankner *et al.*, 1990; Pike *et al.*, 1993; Lorenzo and Yanker, 1994). Aggregation of A β is increased in the presence of ApoE, heparin sulphate proteoglycan, and some heavy metals (Fraser *et al.*, 1992; Strittmatter *et al.*, 1993; Strittmatter *et al.*, 1993; Snow *et al.*, 1994). Multiple molecular mechanism have been proposed to explain the neurotoxic effects of A β including inducing apoptosis both by direct effects on cell membranes and by indirect effects such as potentiating effects of excitatory amino acids, oxidative stress, and cause increases in intracellular calcium and free radicals (Mattson *et al.*, 1992; Arispe *et al.*, 1993; Mattson and Goodman, 1995). However, while much attention has been focused upon the possibility that the neurodegenerative effect of β APP mutations are mediated by overproduction of neurotoxic A β peptide, several other putative mechanisms have emerged. For instance, it has been shown that the APP717 mutations may induce

apoptosis by causing constitutive activation of the heterotrimeric GTP-binding protein G_o (Okamoto *et al.*, 1995; Yamatsuji and Nishimoto, 1996).

Apolipoprotein E

Genetic linkage studies in pedigrees with predominantly late onset familiarly aggregated AD provided suggestive evidence ($z=+2.5$ at $\theta=0.00$) for the existence of a second AD susceptibility locus near the markers *BCL3* and *ATPIA3* which map to chromosome 19q12–q13 (Pericak-Vance *et al.*, 1991). While this initial localization was both crude and tentative, Strittmatter *et al.* isolated proteins from the CSF which were capable of binding the $A\beta$ peptide (Strittmatter *et al.*, 1993). Microsequencing of these peptides revealed that one was apolipoprotein E (ApoE) (Strittmatter *et al.*, 1993). Simultaneously, the presence of ApoE in the senile plaque of AD raised the possibility that ApoE may be a pathological chaperon that affect $A\beta$ aggregation (Wisniewski and Frangione, 1992; Naslund *et al.*, 1995). More significantly, the gene for ApoE maps on chromosome 19q13, very close to the markers showing evidence for linkage and/or association with late onset AD. Cumulatively, these observations clearly raised the possibility that the ApoE gene itself was the AD susceptibility locus.

The ApoE gene in humans contains three common polymorphisms. The most common variant, ϵ_3 , reflects the presence of a cysteine at codon 112, and is present in approximately 75% of Caucasians. A second variant, ϵ_4 , reflects substitution of arginine for cysteine at codon 112, and is present in approximately 15% of Caucasians. The third variant, ϵ_2 , contains cysteine at codons 112 and 158, and is present in approximately 10% of Caucasians. Analysis of these polymorphisms in normal control populations and in patients with AD has consistently shown that there is an increase in the frequency of the ϵ_4 allele in patients with AD (allele frequency in AD is approximately 40%) (Saunders *et al.*, 1993), and there is a smaller reduction in the frequency of the ϵ_2 allele (to about 2% in AD) (Corder *et al.*, 1994). More significantly, there is dose-dependent relationship between the number of copies of ϵ_4 , and the age-of-onset of AD such that ϵ_4/ϵ_4 subjects have an earlier onset than do heterozygous ϵ_4 subjects (Corder *et al.*, 1993). Subjects with an ϵ_2 allele on the other hand have a later onset (Corder *et al.*, 1994). The association between ϵ_4 and AD has been robustly confirmed in numerous studies and in several different ethnic groups (reviewed in Roses, 1996). The association is weaker with advanced age of onset and the putative protective role of the ϵ_2 allele is less clear at younger ages of onset (where it may be associated with a more aggressive course) (Rebeck *et al.*, 1994; Van Duijn *et al.*, 1994). Currently, the single possible exception to the association of ApoE ϵ_4 with AD arises from studies in black Americans which have generated conflicting results (Hendrie *et al.*, 1995; Maestre *et al.*, 1995). It remains unclear whether the conflicting results in black Americans reflect small sample sizes or whether there is a true lack of association between AD and ApoE ϵ_4 in black Americans.

Although with the possible exception of black Americans the association between ApoE ϵ_4 and AD is robust, it is not entirely specific. Observations in patients with head injury (Mayeux and Ottman, 1995; Roses and Saunders, 1995), spontaneous intracerebral haemorrhage (Alberts and Graffagnino, 1995), and in patients undergoing elective cardiac bypass surgery (Newman and Croughwell, 1995), all suggest a poorer outcome for patients with the ϵ_4 allele. There are also conflicting reports of association between the ϵ_4 allele and Creutzfeldt-Jakob disease (Pickering-Brown *et al.*, 1995) and multi-infarct dementia (Shimano *et al.*, 1989), although the majority of studies appear not to detect an

association between $\epsilon 4$ and either of these diseases (Saunders *et al.*, 1993; Salavatore *et al.*, 1995). However, there is a confirmed association between the $\epsilon 4$ allele and the Lewy body variant of AD (Olichney *et al.*, 1996).

The mechanism by which the $\epsilon 4$ allele is associated with an earlier onset of AD, and by which the $\epsilon 2$ allele is associated with a later onset is unclear. The most obvious hypothesis is that ApoE might influence the production, distribution, or clearance of the A β peptide. This hypothesis is supported by observations that the genotype at ApoE accounts for some of the variation in age-of-onset in subjects carrying the β APP Val717Ile mutation (but not the APP₆₉₂ mutation) suggesting a direct biochemical interaction between ApoE and β APP or its metabolic products (St George-Hyslop *et al.*, 1994; van Broeckhoven *et al.*, 1994; Nacmias *et al.*, 1995; Sorbi *et al.*, 1995). Second, subjects with one or more ApoE $\beta 4$ alleles have a higher A β peptide plaque burden than do subjects with no $\epsilon 4$ alleles (Schmechel *et al.*, 1993). *In vitro* studies suggest that delipidated ApoE $\epsilon 4$ binds A β more avidly than ApoE $\epsilon 3$ (Strittmatter *et al.*, 1993; Strittmatter *et al.*, 1993). Finally, there is also evidence that both ApoE and A β may be cleared through the lipoprotein-related (LRP) receptor and that ApoE $\epsilon 4$ and the A β peptide may compete for clearance through the LRP receptor (Kounnas *et al.*, 1994).

While theories attempting to explain a role for ApoE $\epsilon 4$ mediated by alterations in brain A β peptide levels have received the greatest attention, there is also some biochemical evidence to suggest a relationship between ApoE and neurofibrillary tangles and synaptic density. *In vitro* ApoE isoform-specific binding experiments with Tau and MAP2 suggests that $\epsilon 3$ binds to both Tau and MAP2 better than the $\epsilon 4$ isoform (Strittmatter *et al.*, 1994; Huang *et al.*, 1995). This has led to suggestions that $\epsilon 2$ and $\epsilon 3$ may protect and sequester microtubule associated proteins better than $\epsilon 4$ thereby reducing the ability of Tau to bind to itself, become hyperphosphorylated and form paired helical filaments. This is supported by the fact that neurofibrillary degeneration begins earlier in $\epsilon 4$ carriers than non- $\epsilon 4$ carriers (Ohm and Kirca, 1995). Although the ApoE gene is not transcriptionally active in neurons, ApoE immunoreactivity has been detected within the neuronal cytoplasm, and particularly at the base of proximal dendrites suggesting that ApoE can be imported into neurons from extracellular sources (Han and Einstein, 1994). Certainly, the demonstration of intraneuronal ApoE immunoreactivity would place ApoE in the right location for interactions with microtubule associated proteins.

Finally, there is a good correlation between the degree of clinical dementia the decrease in synaptic density in AD as measured by both MAP2 and synaptophysin immunoreactivity (Terry *et al.*, 1994), and it has been suggested that ApoE may be involved in synaptic plasticity during regeneration and repair, and that the $\epsilon 4$ allele is less efficient in this role. Thus, ApoE knockout mice also show an age-dependent decrease in synaptic density and spontaneous A β peptide aggregation within astrocytic processes (Masliah and Mallory, 1995). Several types of neural tissue culture cells demonstrate decreased neurite outgrowth in the presence of ApoE $\epsilon 4$ in the media rather than ApoE $\epsilon 3$ (Nathan *et al.*, 1994; Nathan and Bellosta, 1995).

Presenilin 1

After the discovery that β APP missense mutations were quite rare as a cause of AD, several groups undertook a survey of the remaining non-sex linked chromosomes other than chromosomes 19 and 21. These studies identified a series of polymorphic genetic markers located on chromosome 14q24.3

(D14S43, D14S71, D14S77 and D14S53) which showed robust evidence of linkage to an early onset form of familial Alzheimer disease $z \geq 23.0$ at $\theta=0.01$ (Schellenberg *et al.*, 1992; St George-Hyslop *et al.*, 1992; Van Broeckhoven *et al.*, 1992). Subsequent genetic mapping studies narrowed the region containing this third Alzheimer susceptibility locus (AD3) to a region of approximately 10 centiMorgans between the marker D14S271 at the centromeric end, and D14S53 at the telomeric end, a physical distance of approximately 7 megabases. The definition of flanking markers served to immediately exclude a number of potential candidate genes on chromosome 14. Amongst these genes were the Cathepsin G family of proteins (which mapped closer to the centromere and which might potentially have been involved in β APP processing) and the α -1-anti-chymotrypsin gene (which mapped closer to the telomere and which is a known component of the senile plaque). The cFOS gene and an α -ketoglutarate dehydrogenase subunit gene (E2K or dihydrolipoamide succinyl transferase), both mapped within the minimal cosegregating region, but were excluded by the absence of missense mutations within their open reading frame (Rogaev *et al.*, 1993; Wong *et al.*, 1993). Subsequently, the actual disease gene (presenilin 1) was isolated using the positional cloning strategy as alluded to earlier (Sherrington *et al.*, 1995).

The chromosome 14 AD3 subtype gene, S182 or presenilin 1 (PS1) is highly conserved in evolution, being present in *C. elegans* (Leviton and Greenwald, 1995) and *D. melanogaster* (Boulianne *et al.*, 1997) and appears to encode a polytopic integral membrane protein. Theoretical predictions based upon Kyte-Doolittle hydrophobicity analysis suggest that there are between five and ten membrane spanning domains, that the N-terminus is acidically charged, and that there is a large hydrophilic, acidically charged loop domain between the putative sixth and seventh transmembrane domains (Sherrington *et al.*, 1995; Slunt *et al.*, 1995). Partial direct experimental support for a polytopic structure has been obtained from studies in transfected cells (see below).

The presenilin 1 gene is transcribed at low levels in many different cell types, both within the central nervous system and also in non-neurological tissues (Sherrington *et al.*, 1995). In the CNS PS1 transcripts can be detected by *in situ* hybridization in the neocortex (especially in cortical neurons in layers II and IV), neurons of the CA1-CA3 fields of the hippocampus, granule cell neurons of the dentate gyrus, subiculum, cerebellar Purkinje and granule cells and deep nuclei, as well as lesser amounts in the olfactory bulb, striatum, some brainstem nuclei and thalamus. Despite intense signals on Northern blots of the corpus callosum, there is very little *in situ* hybridization signal detectable in oligodendrocytes in white matter (Cribbs *et al.*, 1996).

The genomic structure of the PS1 gene has been elucidated and some of the transcriptional regulatory elements have been defined (Rogaev *et al.*, 1996). Like the β APP gene there is evidence for alternate splicing of the PS1 transcript. Thus, there is a variably present four-amino acid VRQS insert which arises from use of an alternate splice donor site at the 3' end of exon 4 (The Alzheimer's Disease Collaborative Group, 1995; Cruts *et al.*, 1995; Rogaev *et al.*, 1996). In some tissues (especially leukocytes) there is also alternate splicing of exon 9 which encodes a series of hydrophobic residues at the C-terminus of TM6 and the beginning of the TM6-TM7 exposed loop domain (Rogaev *et al.*, 1996). As a result, this splicing event is predicted to significantly alter the functional properties of the TM6-TM7 loop.

Immunoblotting and immunohistochemical studies suggest that the PS1 protein is approximately 50 kDa in size and is predominantly located within intracellular membranes in the endoplasmic reticulum,

perinuclear envelope, the Golgi apparatus and some as yet uncharacterized intracytoplasmic vesicles (Walter *et al.*, 1996; De Strooper *et al.*, 1997). The subcellular localization of endogenous PS1 is evident, for example, in cultures of human fibroblasts (see Figure 1) which clearly demonstrates the ER/Golgi staining as well as a nuclear envelope component. Similar nuclear staining has recently been reported for PS1 and PS2 (Li *et al.*, 1997) which may be related to a possible role for the presenilins in chromosome organization and/or segregation. The neuronal distribution of PS1 is more widespread with localization to a similar perinuclear domain but also within processes and at the extending growth cones (Figure 1; PEF, PStGH, unpublished data). Studies of the topology of PS1 suggest that the N-terminus and the residues in the TM6-TM7 loop are both located in the cytoplasm (De Strooper *et al.*, 1997). The orientation of the C-terminus is not yet resolved (Thinakaran *et al.*, 1996; De Strooper *et al.*, 1997). Studies of the PS1 protein in brain tissue, as well as many other peripheral tissues, reveal that only very small amounts of the PS1 holoprotein exist within the cell at any given time (Thinakaran *et al.*, 1996; Podlisny *et al.*, 1996). Instead, the holoprotein is actively catabolized, possibly by two different proteolytic mechanisms. One of these mechanisms appears to involve the proteasome, while the other involves endoproteolytic cleavage near residue 290 within the TM6-TM7 loop domain. This endoproteolytic cleavage generates a series of N- and C-terminal heterogeneous fragments of approximately 35 kDa and 18 kDa respectively in size. It is currently unclear whether the holoprotein, the endoproteolytic fragments, or both have biologic functions. The expression patterns of PS1 protein largely reflect those of the mRNA (Uchihara *et al.*, 1996).

The normative function of presenilin 1 has not yet been defined. By analogy to the weakly homologous SPE4 protein of *C. elegans*, which is involved in maintenance of a Golgi derived membranous organelle thought important in partitioning of protein and cell membrane products in the maturing spermatocyte of *C. elegans* (L'Hernault and Arduengo, 1992), it has been speculated that the PS1 protein might subserve a similar role in protein and membrane trafficking (Sherrington *et al.*, 1995). Other putative roles have included a role in the regulation of signal transduction and apoptosis. The former suggestion arose because null mutations in a second presenilin orthologue in *C. elegans* (*sel12*) exerts a suppressor effect on abnormalities in vulva progenitor cell fate decisions induced by activated *Notch* mutants (Levitani and Greenwald, 1995). *Notch* is involved in intercellular signalling in development. SEL 12 protein shows stronger amino acid sequence identity to the human presenilin proteins than does SPE4. A role for mammalian presenilins in Notch mediated is further supported by the fact that homozygous targeted knock-out of the murine PS1 protein using homologous recombination causes embryonic lethality around day E13 and is associated with severe developmental defects in somite formation and axial skeleton formation and the occurrence of cerebral haemorrhage (Wong *et al.*, 1996). Similar phenotypes have been observed in mice with targeted knockouts of the murine Notch 1 gene, thus supporting the hypothesis that PS1 has either a direct or an indirect role in intercellular signal transduction (Conlon *et al.*, 1995). A role in the suppression of apoptosis was suggested by the fact that over-expression of the 3' end of PS2 rescued T-lymphocytes from Fas-ligand induced apoptosis (see below) (Vito *et al.*, 1996). Follow-up studies have to date yielded conflicting evidence as to whether over-expression of full length wild type PS1 or wild type PS2 can cause apoptosis in transfected cells and whether mutations further sensitize these cells to apoptosis (Wolozin *et al.*, 1996). A role for PS2 in apoptosis has been strengthened by the observation of a caspase cleavage which may make cells more vulnerable to apoptotic cell death (Kim *et al.*, 1997).

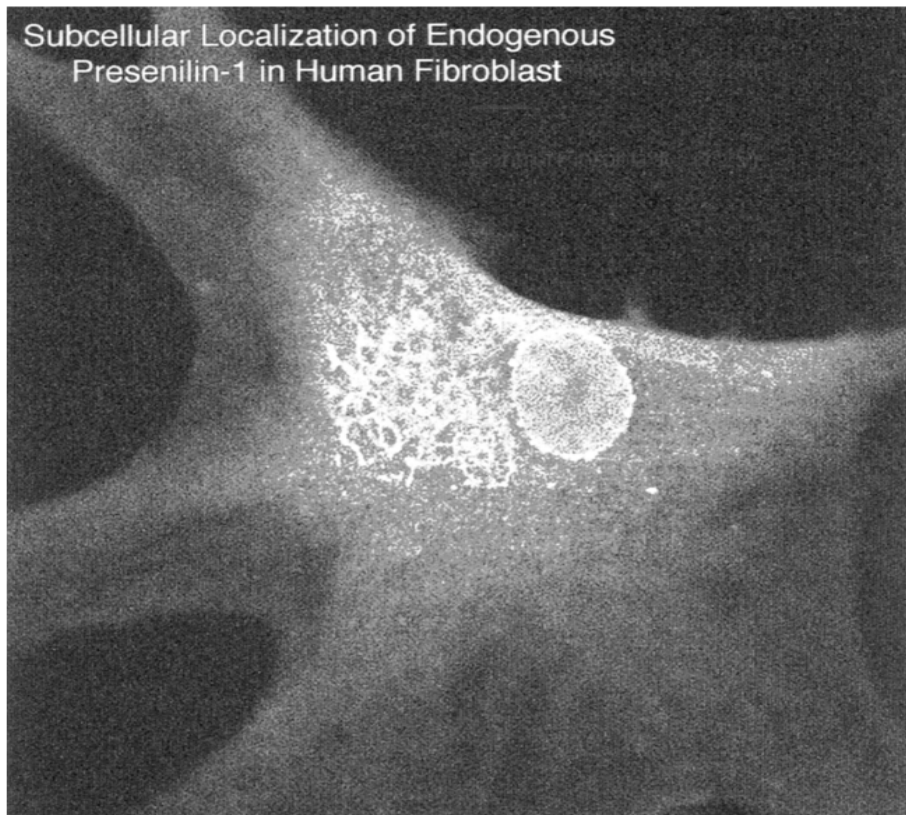


Figure 1. A cultured human fibroblast showing subcellular localization for presenilin-1 wild type protein. Staining was done using an N-terminal antibody specific for PS1 with the highest intensity of labelling indicated in yellow. Perinuclear ER and Golgi staining is apparent as well as punctate staining within the nuclear envelope. (See Colour Plate I)

To date, more than 35 different mutations have been discovered in the PS1 gene (Table 2). The majority of these mutations are missense mutations giving rise to the substitution of a single amino acid. These mutations are predominantly located either in highly conserved transmembrane domains; at or near putative membrane interfaces; or in the N-terminal hydrophobic or C-terminal hydrophobic residues of the putative TM6-TM7 loop domain. A single splicing defect mutation has been identified which involves a point mutation in the splice acceptor site at the 5' end of exon 10 (Perez-Tur *et al.*, 1996; Sato *et al.*, 1996; Kwok *et al.*, 1997). Because exon 9 and exon 11 are in-frame, this mutation allows exon 9 to be fused in-frame with exon 11, thereby removing a series of charged residues at the apex of the hydrophilic acidically-charged TM6-TM7 loop domain. Interestingly, this mutation removes residues near the endoproteolytic cleavage site at residue 290 and results in the production of higher quantities of uncleaved holoprotein (Thinkaran *et al.*, 1996). No deletions, rearrangements or nonsense mutations resulting in truncated proteins which would all cause loss of function mutations

Table 2. Missense mutations in the Presenilin genes.

<i>PRESENILIN 1</i> (Rogaev <i>et al.</i> , 1995; Sherrington <i>et al.</i> , 1995)			
<i>Codon</i>	<i>Location</i>	<i>Mutation</i>	<i>Phenotype</i>
79	N-term loop	Ala->?	FAD onset 64 years CvB personal comm
82	TM1	Val->Leu	FAD onset 55 years (Campion <i>et al.</i> , 1995)
96	TM1	Val->Phe	FAD (Kamino <i>et al.</i> , 1996)
115	TM1->TM2 loop	Tyr->His	FAD onset 37 years (Campion <i>et al.</i> , 1995)
120	TM1->TM2 loop	Glu->Asp	FAD onset 48 years
139	TM2	Met->Thr	FAD onset 49 years (Campion <i>et al.</i> , 1995)
139	TM2	Met->Val	FAD onset 40 years (AD Collaborative Group, 1995)
143	TM2	Ile->Thr	FAD onset 35 years (Cruts <i>et al.</i> , 1995)
146	TM2	Met->Leu	FAD onset 45 years (Sherrington <i>et al.</i> , 1995)
146	TM2	Met->Val	FAD onset 38 years (AD Collaborative Group, 1995)
146	TM2	Met->Ile	FAD onset 40 years
163	TM3 interface	His->Arg	FAD onset 50 years (Sherrington <i>et al.</i> , 1995)
163	TM3 interface	His->Tyr	FAD onset 47 years (AD Collaborative Group, 1995)
171	TM3	Leu->Pro	FAD onset 40 years (Ramirez-Duenas <i>et al.</i> , 1997)
209	TM4 interface	Gly->Val	FAD (Kamino <i>et al.</i> , 1996)
213	TM4 interface	Ile->Thr	FAD (Kamino <i>et al.</i> , 1996)
231	TM5	Ala->Thr	FAD onset 52 years (Campion <i>et al.</i> , 1995)
233	TM5	Met->Thr	FAD onset 35 years (Kwok <i>et al.</i> , 1997)
235	TM5	Leu->Pro	FAD onset 32 years (Campion <i>et al.</i> , 1996)
246	TM6	Ala->Glu	FAD onset 55 years (Sherrington <i>et al.</i> , 1995)
260	TM6	Ala->Val	FAD onset 40 years (Rogaev <i>et al.</i> , 1995)
263	TM6->TM7 loop	Cys->Arg	FAD onset 47 years
264	TM6->TM7 loop	Pro->Leu	FAD onset 45 years (Campion <i>et al.</i> , 1995)
267	TM6->TM7 loop	Pro->Ser	FAD onset 35 years (AD Collaborative Group, 1995)
280*	TM6->TM7 loop	Glu->Ala	FAD onset 47 years (AD Collaborative Group, 1995)
280	TM6->TM7 loop	Glu->Gly	FAD onset 42 years (AD Collaborative Group, 1995)
285	TM6->TM7 loop	Ala->Val	FAD onset 50 years (Rogaev <i>et al.</i> , 1995)
286	TM6->TM7 loop	Leu->Val	FAD onset 50 years (Sherrington <i>et al.</i> , 1995)
Δ291-319	TM6->TM7 loop	short loop	FAD (Perez-Tur <i>et al.</i> , 1996; Sato <i>et al.</i> , 1996; Kwok <i>et al.</i> , 1997)
384	TM6->TM7 loop	Gly->Ala	FAD onset 35 years (Cruts <i>et al.</i> , 1995)
392	TM6->TM7 loop	Leu->Val	FAD onset 25-40 years (Rogaev <i>et al.</i> , 1995)
410	TM7	Cys->Tyr	FAD onset 48 years (Sherrington <i>et al.</i> , 1995)
<i>PRESENILIN II</i>			
141	TM2	Asn->Ile	FAD onset 50-65 years (Levy-Lahad <i>et al.</i> , 1995; Rogaev <i>et al.</i> , 1995)
239	TM5	Met->Val	FAD onset variable 45-84 yrs (Rogaev <i>et al.</i> , 1995)

Mutations in the PS1 and PS2 genes. The majority of the mutations are located in or near putative TM domains. Certain models predict that many of the mutations would be aligned on the internal face of intramembrane helix bundles. The sel 12 Cys60Ser loss of function mutation affects the equivalent Cys92 in PS1. Mutation (denoted by *) creates a more favourable glycosylation site consensus sequence, the Δ291-319 causes a PS1 Exon 10 splicing defect which inhibits physiologic endoproteolytic cleavage.

have yet been found in AD-affected subjects raising the question as to whether such mutations might be lethal or might lead to other disease genotypes.

The effects of PS1 mutations is currently being explored. The wide-scattering of missense mutations, and the absence of null mutations has led to speculation that the effect of the FAD related mutations is a "gain of function" effect (Van Broeckhoven, 1995). This is partially borne out by preliminary studies of gene targeted animals which have loss of functional expression of PS1 protein and in which the phenotype is early perinatal mortality without evidence of Alzheimer disease (Wong *et al.*, 1996). However, it should be noted that preliminary studies using human PS1 cDNAs in complementation assays of mutant sel 12 mutant in *C. elegans* suggest that the wild type human PS1 but not mutant human PS1 cDNAs are able to complement the loss-of-function sel12 mutants (Haass, personal communication). The latter argues that the human PS1 mutants may not be fully functional, but do not preclude a gain of function effect as well. The exact nature of the putative gain of function or loss of function effect imparted by PS1 mutations associated with FAD is unclear. It seems likely that one effect is to alter the processing of β APP by preferentially favouring the production of potentially toxic long-tailed A β peptides ending at residue 42 or 43 (Martin *et al.*, 1995; Duff *et al.*, 1996; Scheuner *et al.*, 1996; Citron *et al.*, 1997). Thus, fibroblasts from heterozygous carriers of PS1 mutations, various cell lines transfected with β APP and PS1 cDNAs, as well as the brain from transgenic mice overexpressing mutant PS1 transgenes all contain or secrete increased quantities of long A β peptide isoforms with only a variable but minor increase in short-tailed A β peptides (Martin *et al.*, 1995; Duff *et al.*, 1996; Scheuner *et al.*, 1996; Citron *et al.*, 1997). Direct measurements of A β peptide isoforms in the post-mortem brain tissue of patients dying with PS1 linked FAD also show marked increases in the amount of long-tailed A β isoforms compared to control brain tissue and to brain tissue from subjects with sporadic Alzheimer disease (Mori *et al.*, unpublished).

As with mutations in the β APP gene, there is a preponderance of evidence pointing to a role for over-production of A $\beta_{x-42(43)}$ as the mechanism underlying neurodegeneration in PS1- and PS2-linked FAD. However, like the β APP mutations evidence has emerged that suggests that PS1 and PS2 mutations may modulate cellular sensitivity to apoptosis induced by a variety of factors including staurosporine, A β peptide or serum withdrawal. At the current time these data are still evolving and the apparent paradox of a putative "apoptosis promoting" effect for the presenilins and the existence of transgenic mice over-expressing mutant or wild-type presenilin cDNAs but lacking widespread apoptosis remains to be explained.

Presenilin 2

During the cloning of the presenilin 1 gene on chromosome 14 a very similar sequence was identified in the public nucleotide sequence databases (Rogaev *et al.*, 1995). Further analysis revealed that this similar nucleotide sequence was derived from a gene on chromosome 1, and encodes a polypeptide whose open reading frame contained 448 amino acids. The sequence of this peptide showed substantial amino acid sequence identity with that of the presenilin 1 protein (overall identity approximately 60%), and would be pre-dicted to have a structural organization very similar to that of PS1 protein. Within the TM domains, the amino acid sequence identity between this new gene and presenilin 1 was even higher (approximately 90%). However, the pattern of transcription of this novel

gene was slightly different from that of presenilin 1 being expressed less homogeneously in the brain and in peripheral tissues where it was maximally expressed in cardiac muscle, skeletal muscle, and pancreas. Finally, when the genomic organization of this novel gene was worked out it was apparent that many of the intron exon boundaries (especially those relating to the highly conserved transmembrane domains) were identical between this gene and presenilin 1 (Levy-Lahad *et al.*, 1996; Sherrington *et al.*, 1996). Cumulatively, these observations therefore suggested that this novel gene, which became known as presenilin 2, was a homologue of the presenilin 1 gene on chromosome 14.

Mutational analyses uncovered two different missense mutations in the presenilin 2 gene in families segregating early-onset forms of Alzheimer disease (Table 2). The first mutation (Asn141Ile) was detected in a proportion of families of Volga German ancestry (Levy-Lahad *et al.*, 1995; Rogaev *et al.*, 1995), in which the FAD locus had been independently mapped by genetic linkage studies to chromosome 1 (Levy-Lahad *et al.*, 1995). The second mutation (Met239Val) was discovered in an Italian pedigree (Rogaev *et al.*, 1995). However, in contrast to the frequency of presenilin 1 mutations, screening of large data sets reveal that presenilin 2 mutations are likely to be rare (Sherrington *et al.*, 1996).

Another profound difference between the presenilin 2 mutations and those in the β APP and PS1 gene is that the phenotype associated with PS2 mutations is much more variable (Sherrington *et al.*, 1996; Bird *et al.*, 1997). Thus, the vast majority of heterozygous carriers of missense mutations in the β APP and PS1 genes develop the illness between the ages of 35 and 65 for PS1 mutations, and between 40 and 65 for β APP mutations. In contrast, the range of age-of-onset in heterozygous carriers of PS2 mutations is between 40 and 85 years of age, and there is at least one instance of apparent non-penetrance in an asymptomatic octogenarian transmitting the disease to affected offspring (Bird, 1988; Bird *et al.*, 1989; Sherrington *et al.*, 1996). A similar, but less profound variation in age-of-onset within families segregating the β APP Val717Ile mutation has been ascribed to a modifying effect by at the ApoE gene (St George-Hyslop *et al.*, 1994; Nacmias *et al.*, 1995; Sorbi *et al.*, 1995). Thus, carriers of the β APP Val717Ile mutation who have one or more ϵ 4 alleles at ApoE have an earlier onset than do heterozygous carriers of the Val717Ile mutation who have the ϵ 2 allele and no ϵ 4 alleles of ApoE. However, because the effect of ApoE ϵ 4 on the age-at-onset in PS2 mutations is either absent or less profound, modifier loci other than ApoE probably account for much of this variation.

The relationship of the normal function of presenilin 2 to that of presenilin 1 remains unknown. There is speculation that PS1 and PS2 may form parts of a hetero-oligomeric complex because both appear to be detected as monomers and as higher molecular weight complexes on Western blots (Walter *et al.*, 1996; Podlisny *et al.*, 1996; Thinkaran *et al.*, 1996), and because both proteins reside within the perinuclear envelope, endoplasmic reticulum, Golgi and some yet uncharacterized intracytoplasmic vesicles. It should be noted, however, that the tissue-specific patterns of expression of PS1 and PS2 appear to be slightly different (Rogaev *et al.*, 1995; Levy-Lahad *et al.*, 1996). Given the strong similarities in structure and in amino acid sequence of the respective proteins, it would seem likely that PS1 and PS2 have similar or overlapping activities. It also seems likely that the effect of PS2 mutations will be similar to those of PS1 mutations because the residues mutated in PS2 are conserved in PS1. In support of this, preliminary data suggest PS2 mutations, like that of PS1 mutations, increase the secretion of long-tailed A β peptides (Scheuner *et al.*, 1996; Citron *et al.*, 1997). PS2

mutations may also cause increased sensitivity to apoptosis, but it is unclear whether this is an effect independent of their ability to cause increased A β peptide secretion.

Other Genes

Several large surveys of subjects with familial Alzheimer disease have indicated that the four currently known AD susceptibility loci do not account for the disease in all pedigrees. Since pedigrees lacking mutations in any of the four known AD genes do not have a singular phenotype, but instead comprise a mix of early-onset autosomal dominant pedigrees and late-onset multiplex pedigrees, it is likely that there are several FAD genes remaining to be found. Some of these FAD loci will probably be associated with rather rare but highly penetrant defects similar to those seen with mutations in PS1 and β APP. Other genes may result in incompletely penetrant autosomal dominant traits like that associated with PS2, while still others may be AD susceptibility genes similar to ApoE.

Several association studies have been performed on likely candidate genes, including α 1-anti-chymotrypsin (Kamboh *et al.*, 1995) and the VDRL receptor (Okuizumi *et al.*, 1995). However, follow-up studies have not generated consistent confirmation of these associations (e.g. no replication was found of the α 1-anti-chymotrypsin association in a large follow-up study (Haines *et al.*, 1996)), and so other loci are currently being sought. Recently, genetic linkage studies in a large data set of pedigrees with late onset AD have provided provisional evidence for the existence of another AD susceptibility locus on chromosome 12.

More recently, a link to butyrylcholinesterase K (BCHE-K) has been reportedly linked to sporadic AD (Lehmann *et al.*, 1997). The allelic frequency of the K variant was significantly higher in these cases as compared to controls, early onset AD or other cases of dementia. In addition, the results from this study indicated that the BCHE-K may be acting in concert with the ApoE4 gene leading to a further risk of AD.

Role of β APP, ApoE and the Presenilins in the Pathogenesis of "Sporadic" AD

The appearance of derivatives of β APP, ApoE (Wisniewski and Frangione, 1992; Schmechel *et al.*, 1993; Wisniewski *et al.*, 1995), and PS1 proteins (Uchihara, *et al.*, 1996; Wisniewski *et al.*, 1996) in pathologic structures in brain tissue of subjects with sporadic AD argue that these proteins are involved in the disease even in cases of AD which are not due to a mutation or polymorphism in these genes. All three of these gene products are present in the amyloid plaque. However, while ApoE is also associated with neurofibrillary tangles and many other amyloids, PS1 and A β are specific to AD associated amyloid deposits, and PS1 appears to bind specifically only to extracellular A β but not to cystatin C or transthyretin. Interestingly, some preliminary reports suggest that in sporadic AD brain, PS1 is expressed in non-tangle bearing neurons (Uchihara *et al.*, 1996; Wisniewski *et al.*, 1996). Further evidence for a role for PS1 in "sporadic" AD derives from the observation that homozygosity for one allele of an intronic polymorphism in the PS1 gene is associated with late onset sporadic AD in some but not all series of AD cases (Scott *et al.*, 1996; Wragg *et al.*, 1996).

Neuropathological and Clinical Phenotypes Associated with Familial Alzheimer's Disease

In general, the overall clinical and pathological phenotypes associated with the early onset autosomal dominant forms of familial Alzheimer disease overlap with, and are indistinguishable from those associated with sporadic Alzheimer disease (with the obvious exception of a younger age-of-onset, and obvious vertical transmission as an autosomal dominant trait) (Goudsmit *et al.*, 1981; Nee *et al.*, 1983; Foncin *et al.*, 1985; Bird *et al.*, 1989; Frommelt *et al.*, 1991; Karlinsky *et al.*, 1992). There are some reports that myoclonus is more prominent in the early onset familial forms of AD, and that the course of the disease is slightly more rapid (Bird *et al.*, 1989). From the neuropathologic perspective, there are occasional cases with somewhat atypical distributions of neurofibrillary tangles, minimal spongiform change in the superficial cortical layers, and rarely some atypical neuronal inclusions (Karlinsky *et al.*, 1992; Lantos *et al.*, 1992; Mann *et al.*, 1992; Lippa *et al.*, 1996; Ikeda *et al.*, 1997). However, none of these clinical or pathological characteristics are pathognomonic of early onset FAD in general or of specific genetic subtypes. It currently remains a tantalizing possibility that the general uniformity of the disease phenotype reflects a common biochemical pathway in which pathological accumulation of the A β peptide plays a central role. If so, it would make FAD an inherited syndrome analogue to the glycogen storage diseases or the mucopolysaccharidoses. However, it is, of course, conceivable that subtle distinguishing differences in the disease phenotype may become apparent once a more robust etiologically based nosologic classification of AD comes to fruition.

ACKNOWLEDGEMENTS

Supported by research grants from the Medical Research Council of Canada, the Canadian Genetic Disease Network, the Alzheimer Association of Ontario, The Ontario Mental Health Foundation, The EJLB Foundation, the Scottish Rite Charitable Trust and the Helen B. Hunter Foundation. We also gratefully acknowledge the collaborative efforts of Drs. Fred van Leuven, Bart De Strooper and Wim Annaert (KU Leuven), Sam Gandy (New York) and Ms. Lyne Lévesque on the study of presenilins in neuronal culture.

REFERENCES

- Alberts, M.J., and Graffagnino, C. (1995) ApoE genotype and survival from intracerebral hemorrhage. *Lancet*, **346**, 575.
- Arispe, N., Pollard, H.B., and Rojas, E. (1993) Giant multilevel cation channels formed by Alzheimer disease amyloid B protein in a bilayer membrane. *Proc. Natl. Acad. Sci. USA.*, **90**, 10573–10577.
- Bergem, A.L.M., Engedal, K., and Kringlen, E. (1992) Twin concordance and discordance for vascular dementia and dementia of the Alzheimer type. *Neurobiol. Aging*, **13**(Suppl. 1), 66.
- Bird, T.D. (1988) Familial Alzheimer's Disease in American descendents of the Volga Germans: probable genetic founder effect. *Ann. Neurol.*, **23**, 25.
- Bird, T.D., Levy-Lehad, E., Poorkaj, J., Nochlin, D., Sumi, S.M., Nemens, E.J., Wijsman, E., and Schellenberg, G.D. (1997) Wide range in age of onset for chromosome 1 related familial AD. *Neurology*, in press.

- Bird, T.D., Sumi, S.M., Nemens, E.J., Schellenberg, G.D., Martin, G., *et al.* (1989) Phenotypic heterogeneity in familial Alzheimer's Disease: a study of 24 kindreds. *Ann. Neurol.*, **25**, 12–25.
- Boulianne, G., Livne-Bar, I., Humphreys, J.M., Rogaev, E., and St George-Hyslop, P. (1997) Cloning and mapping of a close homologue of human presenilins in *D.Melanogaster*. *NeuroReport*, in press :
- Breitner, J.C., Silverman, J.M., Mohs, R.C., and Davis, K.L. (1988) Familial aggregation in Alzheimer Disease: comparison of risk among relatives of early- and late-onset cases, and among male and female relatives in successive generations. *Neurology*, **38**, 207–212.
- Cai, X.D., Golde, T.E., and Younkin, S.G. (1992) Release of excess amyloid beta protein from a mutant beta protein precursor. *Science*, **259**, 514–516.
- Campion, D., Brice, A., Dumanchin, C., Puel, M., Baulac, M., Clerget-Darpoux, F., Agid, Y., and Frebourg, T. (1996) A novel presenilin 1 mutation in familial Alzheimers Disease with onset age of 29 years. *NeuroReport*, **7**, 1582–1584.
- Campion, D., Flaman, J.-M., Brice, A., Hannequin, D., Dubois, B., Martin, C., Moreau, V., Charbonnier, F., Didierjean, O., Tardieu, S., Mallet, J., Bellis M., Clerget-darpoux, C., Agid, Y and Frebourg, T. (1995) Mutations of the Presenilin-1 gene in families with early onset Alzheimer's disease. *Hum. Molec. Genet.*, **4**, 2373–2377.
- Carter, D., Desmerais, E., Bellis, M., Campion, D., Clerget-Darpoux, F., Brice, A., Agid, Y, Jaillard-Seradt, A., and Mallet, J. (1992) More missense in amyloid gene. *Nature Genetics*, **2**, 255–256.
- Chartier-Harlin M-C, Crawford, F, Holden, H., Warren, A., Hughes, D., Fidani, L., Goate, A., Rossor, M., Hardy, J., and Mullan, M. (1991) Early onset Alzheimer's Disease caused by mutations at codon 717 of the B-amyloid gene. *Nature*, **353**, 844–846.
- Church, D.M., Stotler, C.J., Rutter, J.L., Murrell, J.R., Trofatter, J.A., and Buckler, A.J. (1994) Isolation of genes from complex sources of mammalian genomic DNA using exon amplification. *Nature Genetics*, **6**, 98–105.
- Citron, M., Oltersdorf, T., Haass, C, McConlogue, C., Hung A.Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., and Selkoe D.J. (1992) Mutation of the B-amyloid precursor protein in familial Alzheimer's Disease increases B-protein production. *Nature*, **360**, 672–674.
- Citron, M., Westaway, D., Xia, W., Carlson, G., Diehl TS, Levesque, G., Johnson-Wood, K., Lee, M., Seubert, P., Davis, A., Kholodenko, D., Motter, R., Sherrington, R., Perry, B., Yao, H., Strome, R., Lieberburg, L, Rommens, J., Kim, S., Schenk, D., Fraser, P., St George-Hyslop, P., and Selkoe, D.J. (1997) Mutant presenilins of Alzheimer's Disease increase production of 42 residue amyloid B-protein in both transfected cells and transgenic mice. *Nature, Med.* **3**, 67–72.
- Collins, F.S. (1992) Positional cloning: lets not call it reverse anymore. *Nature Genetics*, **1**, 3–6.
- Conlon, R.A., Reaume, A.G., and Rossant, J. (1995) Notchl is required for the coordinate segmentation of somites. *Development*, **121**, 1533–1545.
- Corder, E.H., Saunders, A.M., Risen, N.J., *et al.* (1994) Apolipoprotein, E., type 2 allele decreases the risk of late-onset Alzheimer disease. *Nature Genetics*, **7**, 180–184.
- Corder, E.H., Saunders, A.M., Srittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Small, G.W., Roses, A.D., Haines, J.L., and Pericak-Vance, M.A. (1993) Gene dosage of Apolipoprotein, E., type 4 allele and the risk for Alzheimer's Disease in late onset families. *Science*, **261**, 921–923.
- Cribbs, D.H., Chen, L.S., Bende, S.M., and LaFerla, F.M. (1996) Widespread neuronal expression of the presenilin 1 early onset Alzheimer's Disease gene in murine brain. *Am. J. Pathol.*, **148**, 1797–1806.
- Cruts, M., Martin, J.-J., and Van Broeckhoven, C. (1995) Molecular genetic analysis of familial early-onset Alzheimer's Disease linked to chromosome 14q24.3. *Hum. Molec. Genet.*, **4**, 2363–2371.

- De Strooper, B., Beullens, M., Contreras, B., Craessaerts, K., Moechars, D., Bollen, M., Fraser, P., St George-Hyslop, P., and Van Leuven, F. (1997) Postranslational modification, subcellular localization and membrane orientation of the Alzheimer's Disease associated Presenilins. *J. Biol. Chem.*, **272**, 3590–3598.
- Duff, K., Eckman, C., Zehr, C., Yu, X., Prada, C.H., Pereztur, J., Hutton, M., Refolo, L., Zenk, B., Hardy, J., and Younkin, S. (1996) Increased amyloid beta 42(43) in brains of mice expressing mutant presenilin 1. *Nature*, **383**, 710–713.
- Duyk, G.M., Kim, S., Myers, R.M., and Cox, D.R. (1990) Exon trapping: A., genetic screen to identify transcribed sequences in cloned mammalian genomic DNA. *Proc. Natl. Acad. Sci. USA*, **88**, 4005–4009.
- Farrer, L.A., Myers, R.H., Connor, L., Cupples, L.A., and Growdon, J.H. (1991) Segregation analysis reveals evidence of A., major gene for Alzheimer disease. *Am. J. Hum. Genet.*, **48**, 1026–1033.
- Foncin, J.-F., Salmon, D., Supino-Viterbo, V., Feldman, R.G., Macchi, G., Mariotti, P., Scopetta, C., Caruso, G., and Bruni, A.C. (1985) Alzheimer's Presenile dementia transmitted in an extended kindred. *Rev. Neural. (Paris)*, **141**, 194–202.
- Fraser, P.E., Nguyen, J., Chin, D., and Kirschner, D.A. (1992) Effects of sulphate ions on Alzheimer B/A4 peptide assemblies: implications for amyloid fibril proteoglycan interactions. *J. Neurochem.*, **59**, 1531–1540.
- Frommelt, P., Schnabel, R., Kuhne, W., Nee, L.E., and Polinsky, R.J. (1991) Familial Alzheimer Disease: A., large multigenerational German kindred. *Alzheimer Dis. Assoc. Disorders*, **5**, 36–43.
- Goate, A., Haynes, A.R., Owen, M.J., Farrall, M., James, L.A., Lai, L.Y., Mullan, M., Rossor, M., and Hardy, J. (1989) Predisposing locus for Alzheimer Disease on chr 21. *Lancet*, **1**, 352–355.
- Goate, A.M., Chartier-Harlin, M.-C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Guiffra, L., Haynes, A., and Hardy, J.A. (1991) Segregation of A., missense mutation in the amyloid precursor protein gene with Familial Alzheimer Disease. *Nature*, **349**, 704–706.
- Goldgaber, D., Lerman, M.I., McBride, O.W. *et al.* (1987) Characterization and chromosomal localization of a cDNA encoding brain amyloid of Alzheimer's Disease. *Science*, **235**, 877–880.
- Goudsmit, J., White, B.J., Weitkamp, L.R., Keats, B.J., Morrow, C.H., and Gadjusek, D.C. (1981) Familial Alzheimer's Disease in two kindreds of the same geographic origin: A clinical and genetic study. *J. Neurol Sci.*, **49**, 78–89.
- Gusella, J.F. (1986) DNA polymorphisms and human disease. *Ann. Rev. Biochem.*, **55**, 831–834.
- Haas, C., Lemere, C., Capell, A., Citron, M., and Selkoe, D. (1995) The Swedish mutation causes early onset Alzheimer's disease by B-secretase cleavage within the secretory pathway. *Nature Med.*, **1**, 1291–1296.
- Haass, C., Hung, A.Y., Selkoe, D.J., and Teplow, D.B. (1994) Mutations associated with a locus for familial Alzheimer's Disease result in alternative processing of amyloid B-protein precursor. *J. Biol. Chem.*, **269**, 17741–17748.
- Haines, J.L., Pritchard, M.L., Saunders, A.M., Schickkraut, J.M., Growdon, J.H., Gaskell, P., Farrer, L.A., Auerbach, S.A., Gusella, J.F., Yamoaka, L., Conneally, P.M., Roses, A.D., and Pericak-Vance, M.A. (1996) No genetic effect of alpha-1 antichymotrypsin in Alzheimer Disease. *Genomics*, **33**, 53–56.
- Han, S.H. and Einstein, G. (1994) Apolipoprotein, E., is localized to the cytoplasm of human cortical neurons: A light and electron microscopic study. *J. Neuropathol. Exp. Neurol.*, **53**, 535–544.
- Hendricks, M., van Duijn, C.M., Cras, P., Cruts, M., van Hul, W., Van Harskamp, F., Warren, A., McInnis, M., Antonarakis, G., Martin, J.-J., Hofman, A., and Van Broeckhoven, C. (1992) Presenile dementia and cerebral hemorrhage linked to a mutation at codon 692 of the B-amyloid Precursor protein gene. *Nature Genetics*, **1**, 218–221.

- Hendrie, H.C., Hall, K.S., and Hui, S. (1995) Apolipoprotein, E., genotypes and Alzheimer's Disease in a community study of elderly African Americans. *Ann. Neurol.*, **37**, 118–121.
- Heyman, A., Wilkinson, W.E., Stafford, J.A., Helms, M.J., Sigmon, A.H., and Weinberg, T. (1984) Alzheimer's Disease: a study of epidemiological aspects. *Ann. Neurol.*, **15**, 335–341.
- Huang, D.K., Weisgraber, K.H., Strittmatter, D., Goldgaber, D., and Roses, A.D. (1995) ApoE3 binding to tau tandem repeat I is abolished by tau serine262 phosphorylation. *Neurosci. Lett.*, **192**, 209–212.
- Huang, D.Y., Goedert, M., Strittmatter, W., Schmechel, D., Weisgraber, K., and Roses, A.D. (1995) Isoform-specific interactions of apolipoprotein E with microtubule associated protein MAP2c: implications for Alzheimer Disease. *Neurosci. Lett.*, **182**, 55–58.
- Hyman, B.T., West, H.L., Rebeck, G.W., Lai, F., and Mann, D.M.A. (1994) Neuropathological changes in Down syndrome hippocampal formation: effect of age and Apolipoprotein E genotype. *Arch. Neurol.*, in the press.
- Ikedo, M., Sharma, V., Sumi, S.M., Rogaeva, E., Poorkaj, P., Sherrington, R., Nee, L., Tsuda, T., Oda, N., Watanabe, M., Aoki, M., Hirai, S., Schellenberg, G.D., Bird, T.D. and St George-Hyslop, P.H. (1997) The clinical phenotype of two missense mutations in the presenilin 1 gene in Japanese patients. *Ann. Neurol.*, **40**, in press.
- Jarrett, J.T., and Lansbury, P.T. (1993) Seeding one-dimensional crystallization of amyloid: a patho-genic mechanism in Alzheimer's Disease and scrapie? *Cell*, **73**, 1055–1058.
- Jones, C.T., Morris, S., Yates, C.M., Moffoot, A., Sharpe, C., Brock, D.J., and St Clair, D. (1992) Mutation in codon 713 of the beta amyloid precursor protein gene presenting with schizophrenia. *Nature Genet.*, **1**, 306–309.
- Kamboh, M.I., Sanghera, D.K., Ferrell, R.E., and DeKosky, S.T. (1995) ApoE ϵ 4 associated Alzheimer's Disease risk is modified by alpha 1 anti-chymotrypsin polymorphism. *Nature Genet.*, **10**, 486–488.
- Kamino, K., Orr, H.T., Payami, H., Wijsman, E.M., Alonso, M.E., Anderson, L., O'dahl, S., Nemens, E., and White, J. (1992) Linkage and mutational analysis of FAD kindreds for the APP region. *Am. J. Hum. Genet.*, **51**, 998–1014.
- Kamino, K., Sato, S., Sakaki, Y., Yoshiiwa, A., Nishiwaki, Y., Takeda, M., Tanabe, H., Nishimura, T., Li, K., St George-Hyslop, P., Miki, T., and Ohihara, T. (1996) Three different mutations of the presenilin 1 gene in early onset Alzheimer's Disease families. *Neurosci. Lett.*, **208**, 195–198.
- Kang, J., Lemaire, H.G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Multhap, G., Beyreuther, K., and Muller-Hill, B. (1987) The precursor of Alzheimer disease amyloid A4 protein resembles a cell surface receptor. *Nature*, **325**, 733–736.
- Karlinsky, H., Vaula, G., Haines, J.L., Ridgley, J., Bergeron, C., Mortilla, M., Tupler, R., Percy, M., Robitaille, Y., Crapper MacLachlan, D.R., and St George-Hyslop, P. (1992) Molecular and prospective phenotypic characterization of a pedigree with Familial Alzheimer Disease and a missense mutation in codon 717 of the B-Amyloid Precursor Protein (APP) gene. *Neurology*, **42**, 1445–1453.
- Katzman, R., and Kawas, C. (1994). *The epidemiology of dementia and Alzheimer Disease. Alzheimer Disease*. New York, Raven Press. 105–122.
- Kim, T.-W., Pettingell, W.H., Jung, Y.-K., Kovacs, D.M., and Tanzi, R.E. (1997) *Science*, **277**, 373
- Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S., and Ito, H. (1988) Novel precursor of Alzheimer's Disease amyloid protein shows protease inhibitory activity. *Nature*, **331**, 530–532.
- Kounnas, M.Z., Moir, R.D., Rebeck, G.W., Bush, A.I., Argaves, W., Hyman, B.T., and Strickland, D.K. (1994) LDL Receptor related protein, a multifunctional ApoE receptor binds secreted β APP and mediates its degradation. *Cell*.
- Kwok, J.B., Tadder, K., Fisher, C., Hallup, M., Brooks, W., Nicholson, G., St George-Hyslop, P., Fraser, P.E., Relkin, N., Gandy, S.E., Schofield, R., and Martins, R. (1997) Sequence analysis of presenilin genes in early-onset Alzheimer Disease reveals a novel (Met233Thr) presenilin 1 mutation.: submitted.

- L'Hernault, S.W.L., and Arduengo, P.M. (1992) Mutation of a putative sperm membrane protein in *Caenorhabditis elegans* prevents sperm differentiation but not its associated meiotic divisions. *J. Cell Biol.*, **119**, 55–69.
- Lantos, P.L., Luthert, P.J., Hanger, D., Anderton, B.H., Mullan, M., and Rossor, M. (1992) Familial Alzheimer disease with APP position 717 mutation and sporadic AD have the same cytoskeletal pathology. *Neurosci. Letts.*, **137**, 221–224.
- Lautenschlager, N.T., Cupples, L.A., Rao, V.S., Auerbach, S.A., Becker, R., Burke, J., Chui, H., Duara, R., Volicer, L., Waring, S.C., Growdon, J.H., and Farrer, L.A. (1996) Risk of dementia among relatives of Alzheimer Disease patients in the MIRAGE study: what is in store for the oldest old? *Neurology*, **46**, 641–650.
- Lehman, D.J., Johnston, C., and Smith, A.D. (1997) Synergy between the genes for butyrylcholinesterase K variant and apolipoprotein $\epsilon 4$ in late-onset confirmed Alzheimer's disease. *Human Mol. Gen.* **6**, 1933–1936.
- Levitan, D., and Greenwald, I. (1995) Facilitation of lin-12-mediated signalling by sel-12, a *Caenorhabditis elegans* S182 Alzheimer's Disease gene. *Nature*, **377**, 351–354.
- Levy, E., Carman, M.D., Fernandez-Madrid, I.J., Power, M.D., Lieberburg, I., Sjoerd, G., van Duinen, S.G., Bots, G., Luyendijk, W., and Frangione, B. (1990) Mutation of the Alzheimer's Disease amyloid gene in hereditary cerebral hemorrhage—Dutch type. *Science*, **248**, 1124–1126.
- Levy-Lahad, E., *et al.* (1995) a familial Alzheimer's Disease locus on chromosome 1. *Science*, **269**, 970–973.
- Levy-Lahad, E., Poorkaj, P., Wang, K., Fu, Y.H., Oshima, J., Mulligan, J., and Schellenberg, G.D. (1996) Genomic structure and expression of STM2, the chromosome 1 Familial Alzheimer Disease gene. *Genomics*, **34**, 198–204.
- Li, J., Xu, M., Zhou, H., Jianyl, M., and Potter, H. (1997) Alzheimer presenilins in the nuclear membrane, interphase kinetochores, and centrosomes suggest a role in chromosome segregation. *Cell*, **90**, 917–927
- Lippa, C.F., Saunders, A.M., Smith, T.W., Swearer, J.M., Drachman, D.A., Ghetti, B., Nee, L., Pulaski-Salo, D., Dickson, D., Robitaille, Y., Bergeron, C., Crain, B., Benson, M., Farlow, M., Hyman, B.T., St George-Hyslop, P., Roses, A.D., and Pollen, D.A. (1996) Familial and sporadic Alzheimer's disease: neuropathology cannot exclude a final common pathway. *Neurology*, **46**, 406–412.
- Lorenzo, A., and Yanker, B.A. (1994) B-amyloid neurotoxicity requires fibril formation and is inhibited by Congo red. *Proc. Natl. Acad. Sci. USA*, **91**, 12243–12247.
- Maestre, G., Ottman, R., Stern, Y., and Mayeux, R. (1995) Apolipoprotein E and Alzheimer Disease: ethnic variation in genotype risks. *Ann. Neurol.*, **37**, 254–259.
- Mann, D.M.A., Jones, D., Snowden, J.S., Neary, D., and Hardy, J. (1992) Pathological changes in the brain of a patient with FAD having a missense mutation in codon 717 of the APP gene. *Neurosci. Lett.*, **137**, 225–228.
- Martin, R.N., Turner, B.A., Carroll, R.T., Sweeney, D., Kim, K.S., Wisniewski, H.M., Blass, J.P., Gibson, G.E., and Gandy, S.E. (1995) High levels of amyloid beta-protein from S182 (Glu246) familial Alzheimer's cells. *NeuroReport*, **7**, 217–220.
- Masliah, E., and Mallory, M. (1995) Neurodegeneration in the central nervous system of ApoE deficient mice. *Exp. Neurol.*, **136**, 107–122.
- Mattson, M.P., Cheng, B., Davis, D., Bryant, K., Lieberburg, I., and Rydel, R. (1992) Beta-amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *J. Neurosci.*, **12**, 376–389.
- Mattson, M.P., and Goodman, Y. (1995) Different amyloidogenic peptides share a similar mechanism of neurotoxicity involving reactive oxygen species and calcium. *Brain Res.*, **676**, 219–224.
- Mayeux, R., and Ottman, R. (1995) Synergistic effects of traumatic head injury and ApoE $\epsilon 4$ in patients with Alzheimer's Disease. *Neurology*, **45**, 555–557.

- Milward, A.E., Papadopoulos, R., Fuller, S.J., Moir, R.D., Small, D., Beyreuther, K., and Masters, C.L. (1992) The amyloid protein precursor of Alzheimer Disease is a mediator of the effects of NGF on neurite outgrowth. *Neuron*, **9**, 129–137.
- Mullan, M.J., Crawford, F., Axelman, K., Houlden, H., Lilius, L., Winblad, B., Lannfelt, L., and Hardy, J. (1992) a pathogenic mutation for probable Alzheimer's Disease in the APP gene at the N-terminus of B-amyloid. *Nature Genetics*, **1**, 345–347.
- Murrell, J., Farlow, M., Ghetti, B., and Benson, M.D. (1991) a mutation in the amyloid precursor protein associated with hereditary Alzheimer's Disease. *Science*, **254**, 97–99.
- Nacmias, B., Latteraga, S., Tulen, P., Piacentini, S., Bracco, L., Amaducci, L., and Sorbi, S. (1995) ApoE genotype and familial Alzheimer's Disease: a possible influence on age-of-onset in APP717Val->Ile mutated families. *Neurosci. Letts.*, **183**, 1–3.
- Naruse, S., Igarashi, S., Kobayashi, H., Aoki, K., Inuzuki, I., Kaneko, K., Shimizu, T., Iihara, K., Kojima, T., Miyatake, T., and Tsuji, S. (1991) Missense mutation (Val->Ile) in exon 17 of the amyloid precursor protein gene in Japanese familial Alzheimer Disease. *Lancet*, **337**, 978–979.
- 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., and Bellosta, S. (1995) The inhibitory effect of apolipoprotein E ϵ 4 on neurite outgrowth is associated with microtubule depolymerization. *J. Biol. Chem.*, **270**, 19791–19799.
- Nathan, B.P., Bellosta, S., Sanan, D.A., Weisgraber, K.H., Mahley, R.W., and Pitas, R.E. (1994) Differential effects of Apolipoproteins ϵ 3 and ϵ 4 on neuronal growth *in vitro*. *Science*, **264**, 850–852.
- Nee, L., Polinsky, R.J., Eldridge, R., Weingartner, H., Smallberg, S., and Ebert, M. (1983) a family with histologically confirmed Alzheimer Disease. *Arc. Neurol.*, **40**, 203–208.
- Newman, M.F., and Croughwell, N.D. (1995) Predictors of cognitive decline after cardiac operation. *Ann. Thorac. Surg.*, **59**, 1326–1330.
- Nishimoto, I., Okamoto, T., Matsuura, Y., Takahashi, S., Murayama, Y., and Ogata, F. (1993) Alzheimer amyloid protein precursor complexes with brain GTP-binding protein Go. *Nature*, **362**, 75–79.
- Nisson, P.E., Hadley, R.G., and Watkins, P.C. (1993) Finding and cloning genes directly from complex DNA by exon trapping. *Focus*, **15**, 26–31.
- Ohm, T.G., and Kirca, M. (1995) Apolipoprotein E polymorphism influences not only cerebral senile plaque load but also Alzheimer type neurofibrillary degeneration. *Neuroscience*, **66**, 583–587.
- Okamoto, T., Takeda, S., Murayama, Y., Ogata, E., and Nishimoto, I. (1995) Ligand-dependent G protein coupling function of amyloid transmembrane precursor. *J. Biol. Chem.*, **270**, 4205–4208.
- Okuizumi, K., Onodera, O., Namba, Y., Ikeda, K., Yamamoto, T., Seki, K., Ueki, A., Nanko, S., Tanaka, H., Takahashi, H., and Tsuji, S. (1995) Genetic association of the very low density lipoprotein (VLDL) receptor gene with sporadic AD. *Nature Genet.*, **11**, 207–209.
- Olichney, J.M., Hansen, L.A., Galasko, D., Saitoh, T., Hofstetter, C.A., Katzman, R., and Thal, L.J. (1996) The ApoE ϵ 4 allele is associated with increased neuritic plaques and cerebral amyloid angiopathy in AD and in Lewy Body Variant. *Neurology*, **47**, 190–196.
- Peacock, M., J.T., W., Roses, A.D. and Fink, J.K. (1992) Novel polymorphism in the A4-region of the Amyloid Precursor Protein Gene in a patient without Alzheimer's Disease. *Neurology*, **43**, 1254–1256.

- Peacock, M.L., Murman, D.L., Sima, A.A.F., Warren, J.T., Roses, A.D., and Fink, J.K. (1994) Novel amyloid precursor protein gene mutation (codon 665Asp) in a patient with late-onset Alzheimer Disease. *Ann. Neurol.*, **35**, 432–438.
- Perez-Tur, J., Froelich, S., Prihar, G., Crook, R., Baker, M., Duff, K., Wragg, M., Hardy, J., Goate A Lannfelt, L., and Hutton, M. (1996) a mutation in Alzheimer's disease destroying a splice acceptor site in the presenilin 1 gene. *NeuroReport*, **7**, 297–301.
- Pericak-Vance, M.A., Bedout, J.L., Gaskell, P.C., and Roses, A.D. (1991) Linkage studies in Familial Alzheimer Disease—evidence for chromosome 19 linkage. *Am. J. Hum. Genet.*, **48**, 1034–1050.
- Pickering-Brown, S.M., Mann, D.M., Owen, F., Ironside, J.W., de Silva, R., Roberts, D.A., Balderson, D.J., and Cooper, P.N. (1995) Allelic variations in Apolipoprotein E and prion protein genotype related to plaque formation and age of onset in sporadic Creutzfeldt-Jacob Disease. *Neurosci. Lett.*, **187**, 127–129.
- Pike, C.J., Burdick, D., Walencewicz, A.J., Glabe, C.G., and Cotman, C.W. (1993) Neurodegeneration induced by beta-amyloid peptides *in vitro*: the role of peptide assembly state. *J. Neurosci.*, **13**, 1676–1678.
- Ramirez-Duenas, M.G., Rogaeva, E., Leal, C.A., C, L., Ramirez-Casillasm, G.A., Hernandez-Romo, J.A., St George-Hyslop, P.H., and Cantu, J.M. (1997) a novel mutation in presenilin 1 gene in a mexican family with early onset Alzheimer Disease.: submitted.
- Rebeck, G.W., Perls, T., West, H., Sodhi, P., Lipsitz, L.A., and Hyman, B.T. (1994) Reduced apolipoprotein $\epsilon 4$ allele in the oldest old Alzheimer's patients and cognitively normal individuals. *Neurology*, in the press.
- Robakis, N.K., Lahiri, D.K., Brown, H.R., Rubenstein, R., Mehta, B., Wisniewski, H., and Goller, N. (1988). Expression studies of the gene encoding the Alzheimer's Disease and Down Syndrome amyloid peptide. Disorders of the Developing Nervous System: changing views on their origins, diagnoses and treatments. New York, Alan, R., *Liss.*, 183–193.
- Rocca, W.A., Amaducci, L.A., and Schoenberg, B.S. (1986) Epidemiology of clinically diagnosed Alzheimer's Disease. *Ann. Neurol.*, **19**, 415–424.
- Rogaev, E.I., Lukiw, W.J., Vaula, G., Haines, J.L., Rogaeva, E.A., Tsuda, T., Alexandrova, N., and St George-Hyslop, P. (1993) Analysis of the cFOS gene on chr 14 and the 5' promoter of the Amyloid Precursor Protein gene in Familial Alzheimer's Disease. *Neurology*, **43**, 2275–2279.
- Rogaev, E.I., Sherrington, R., Rogaeva, E.A., Levesque, G., Ikeda, M., Liang, Y., Chi, H., Lin, C., Holman, K., Tsuda, T., Mar, L., Sorbi, S., Nacmias, B., Piacentini, S., Amaducci, L., Chumakov, I., Cohen, D., Lannfelt, L., Fraser, P.E., Rommens, J.M., and St George-Hyslop, P. (1995) Familial Alzheimer's disease in kindreds with missense mutations in a novel gene on chromosome 1 related to the Alzheimer's Disease type 3 gene. *Nature*, **376**, 775–778.
- Rommens, J.M., Lin, B., Hutchinson, G.B., Andrew, S.E., Goldberg, Y.P., Glaves, M.L., Graham, R., Lai, V., McArthur, J., Nasir, J., Thellman, J., McDonald, H., Kalchman, M., Clarke, L.A., Schappert, K., and Hayden, M.R. (1993) A transcription map of the region containing the Huntington Disease gene. *Hum. Molec. Genet.*, **2**, 901–907.
- Roses, A.D. (1996) Apolipoprotein E alleles as risk factors in Alzheimer disease. *Annu. Rev. Med.*, **47**, 387–400.
- Roses, A.D., and Saunders, A.M. (1995) Head injury, amyloid B and Alzheimers Disease. *Nature, Med.* **1**, 603–604.
- Royston, M.C., Mann, D., Pickering-Brown, S., Owen, F., Ragbavan, R., Khin-Nu, C., Day, K., Crook, R., Hardy, J., and Roberts, G.W. (1996) ApoE $\epsilon 2$ allele, Down's syndrome and dementia. *Ann. N.Y. Acad. Sci.*, **777**, 255–259.
- Rumble, B., Retallack, R., and Hilbich, C. (1989) Amyloid A4 protein and its precursor in Down's syndrome and Alzheimer's disease. *N. Eng. J. Med.*, **320**, 1446–1452.
- Saitoh, T., Sundsmo, M., Roch, J.-M., Kimura, N., Cole, G., Schubert, D., Oltersdorf, T., and Schenk, D.B. (1989) Secreted form of amyloid B-protein is involved in the growth regulation of fibroblasts. *Cell*, **58**, 615–622.

- Salavatore, M., Seeber, A.C., Nacmias, B., Petraroli, R., D'Assandro, M., Sorbi, S., and Pocchiari, M. (1995) Apolipoprotein E in sporadic and Familial Creutzfeldt-Jacob Disease. *Neurosci. Letts.*, **199**, 95–98.
- Sato, S., Kamino, K., Miki, T., Doi, A., Li, K., St George-Hyslop, P., Ogihara, T., and Sakaki, Y. (1996) Splicing mutation of presenilin 1 gene for early onset familial Alzheimer's Disease. *Hum. Mutation*, in press.
- Saunders, A., Strittmatter, W.J., Schmechel, S., St George-Hyslop, P., Pericak-Vance, M., Joo, S.H., Rosi, B.L., Gusella, J.F., Crapper-McLachlan, D., Growden, J., Alberts, M.J., Hulette, C., Crain, B., Goldgaber, D., and Roses, A.D. (1993) Association of Apolipoprotein E allele $\epsilon 4$ with the late-onset familial and sporadic Alzheimer Disease. *Neurology*, **43**, 1467–1472.
- Saunders, A.M., Schmechel, K., Breitner, J.C.S., Benson, D., Brown, W.T., Goldfarb, L., Goldgaber, D., Manwaring, M.G., Pericak-Vance, M., and Roses, A.D. (1993) Apolipoprotein E $\epsilon 4$ allele distribution in late onset AD and in other amyloid forming diseases. *Lancet*, **342**, 710–711.
- Schellenberg, G.D., Bird, T.D., Wijsman, E.M., Orr, H.T., Anderson, L., Nemens, E., White, J.A., and Bonnycastle, L. (1992) Genetic Linkage evidence for a familial Alzheimer's Disease locus on chr 14. *Science*, **258**, 668–670.
- Scheuner, D., Eckman, L., Jensen, M., Sung, X., Citron, M., Suzuki, N., Bird, T., Hardy, J., Hutton, M., Lannfelt, L., Selkoe, D., and Younkin, S. (1996) Secreted amyloid- β protein similar to that in the senile plaques of Alzheimer Disease is increased *in vivo* by presenilin 1 and 2 and APP mutations linked to FAD. *Nature Med.*, **2**, 864–870.
- Schmechel, D.E., Saunders, A.M., Strittmatter, W.J., Joo, S.H., Hulette, C., Crain, B., Goldgaber, D., and Roses, A.D. (1993) Increased vascular and plaque β -A4 amyloid deposits in sporadic Alzheimer Disease patients with apolipoprotein $\epsilon 4$. *Proc. Natl. Acad. Sci. USA*, **90**, 9649–9653.
- Schupf, N., Kapell, D., Lee, J.H., Zigman, W., Canto, B., Tycko, B., and Mayeux, R. (1997) Onset of dementia is associated with Apolipoprotein E $\epsilon 4$ in Down syndrome. *Ann. Neurol.*, in press.
- Scott, W.K., Growdon, J.H., Roses, A.D., Haines, J.L., and Pericak-Vance, M. (1996) Presenilin 1 polymorphism and Alzheimer Disease. *Lancet*, **347**, 1186–1187.
- Selkoe, D.J. (1994) Normal and abnormal biology of B-Amyloid Precursor Protein. *Ann. Rev. Neurosci.*, **17**, 489–517.
- Sherrington, R., Froelich, S., Sorbi, S., Campion, D., Chi, H., Rogaeva, E.A., Levesque, G., Rogaev, E.I., Lin, C., Liang, Y., Ikeda, M., Mar, L., Brice, A., Agid, Y., Percy, M.E., Clerget-Darpiux, F., Karlinsky, H., Piacentini, S., Marcon, G., Nacmias, B., Amaducci, L., Frebourg, T., Lannfelt, L., Rommens, J.M., and St George-Hyslop, P.H. (1996) Alzheimer's disease associated with mutations in presenilin-2 are rare and variably penetrant. *Hum. Molec. Genet.*, **5**, 985–988.
- Sherrington, R., Rogaev, E., Liang, Y., Rogaeva, E., Levesque, G., Ikeda, M., Chi, H., Lin, C., Holman, K., Tsuda, T., Mar, L., Fraser, P., Rommens, J.M., and St George-Hyslop, P. (1995) Cloning of a gene bearing missense mutations in early onset familial Alzheimer's disease. *Nature*, **375**, 754–760.
- Shimano, H., Ishibashi, S., Murase, T., Gotohda, T., Yamada, N., Takahu, F., and Ohtomo, E. (1989) Plasma apolipoproteins in patients with multi-infarct dementia. *Atherosclerosis*, **79**, 257–260.
- Slunt, H.H., Thinkaran, G., Lee, M.K., and Sisodia, S.S. (1995) Nucleotide sequence of the chromosome 14 encoded S182 cDNA and revised secondary structure prediction. *Amyloid*, **2**, 188–190.
- Snow, A.D., Sekiguchi, R., Nochlin, D., Fraser, P., Kimata, K., Arai, M., Schreier, W.A., and Morgan, D.A. (1994) An important role of heparan sulfate proteoglycan in a model system for the deposition and persistence of fibrillar A β -amyloid in rat brain. *Neuron*, **12**, 219–234.
- Sorbi, S., Nacmias, B., Forleo, P., and Amaducci, L. (1995) Epistatic effect of APP717 mutation and apolipoprotein E genotype in familial Alzheimer Disease. *Ann. Neurol.*, **38**, 124–128.

- St George-Hyslop, P., Haines, J., Rogaev, E., Mortilla, M., Vaula, G., Pericak-Vance, M., Foncin, J.-F., Montesi, M., Bruni, A., Sorbi, S., Rainero, I., Pinessi, I., Pollen, D., Polinsky, R., Nee, L., Kennedy, J., Macciardi, F., Rogeava, E., Liang, Y., Alexandrova, N., Lukiw, W., Schlumpf, K., Tsuda, T., Farrer, L., Cantu, J.-M., Duara, R., Amaducci, L., Bergamini, L., Gusella, J., Roses, A., and Crapper MacLachlan, D. (1992) Genetic Evidence for a novel Familial Alzheimer Disease gene on chromosome 14. *Nature Genetics*, **2**, 330–334.
- St George-Hyslop, P., Polinsky, R., Haines, J., Nee, L., Tanzi, R., Conneally, P., Growdon, J., Myers, R., Pollen, D., Drachman, D., Feldman, R., Amaducci, L., Foncin, J.-F., Bruni, A., Frommelt, P., and Gusella, J. (1987) Search for the Familial Alzheimer Disease gene. *J. Neural Transm.*, **24**(suppl.), 13–21.
- St George-Hyslop, P.H., Haines, J.L., Farrer, L.A., van Broeckhoven, C., Goate, A.M., CrapperMcLachlan, D.R., *et al.* (1991) Genetic Linkage studies suggest that Alzheimer's Disease is not a single homogeneous disorder. *Nature*, **347**, 194–197.
- St George-Hyslop, P.H., Tsuda, T., Crapper McLachlan, Karlinsky, H., Pollen, D., and Lippa, C. (1994) Alzheimer's Disease and possible gene interaction. *Science*, **263**, 536–537.
- Strittmater, W.J., Saunders, A.M., Goedert, M., Weisgraber, K.H., Dong, L.M., Jakes, R., Huang, D., Pericak-Vance, M., Schmechel, D., and Roses, A. (1994) Isoform specific interactions of ApoE with microtubule associated protein Tau: implications for Alzheimer Disease. *Proc. Natl. Acad. Sci. USA*.
- Strittmatter, W.J., Saunders, A.M., Schmechel, D., Goldgaber, D., Roses, A.D., *et al.* (1993) Apolipoprotein E: high affinity binding to B/A4 amyloid and increased frequency of type 4 allele in Familial Alzheimers Disease. *Proc. Natl Acad. Sci. USA*, **90**, 1977–1981.
- Strittmatter, W.J., Weisgraber, K.H., Huang, D.Y., Schmechel, D., Saunders, A.M., and Roses, A.D. (1993) Binding of human lipoprotein E to synthetic amyloid beta peptide: isoform-specific effects and implications for late onset AD. *Proc. Natl. Acad. Sci. USA*, **90**, 8098–8102.
- Susuki, N., Cheung, T.T., Cai, X.-D., Odaka, A., Otvos, L., Eckman, C., Golde, T., and Younkin, S.G. (1994) An increased percentage of long amyloid B protein secreted by Familial Amyloid B Protein Precursor (BAPP717) mutants. *Science*, **264**, 1336–1340.
- Tagle, D.A. and Collins, F.S. (1992) An optimized Alu-PCR primer pair for human specific amplification of YACs and somatic cell hybrids. *Hum. Molec. Genet.*, **1**, 121–122.
- Tanzi, R.E., Gusella, J.F., Watkins, P.C., Bruns, G.A.P., St George-Hyslop, P.H., and Van Keuren, M.L. (1987) Amyloid B-protein gene: cDNA, mRNA distribution and genetic linkage near the Alzheimer locus. *Science*, **235**, 880–884.
- Terry, R.D., Masliah, E., and Hansen, L. (1994). Structural basis of the cognitive alterations in Alzheimer. *Alzheimer Disease*. New York, Raven Press. pp. 179–196.
- The Alzheimer's Disease Collaborative Group (1995) The structure of the presenilin, I, gene and the identification of six mutations in early onset AD pedigrees. *Nature Genet.*, **11**, 219–222.
- The French Alzheimer's Disease Study Group (1996) No founder effect in three novel Alzheimer's Disease families with APP 717 Val Ile mutation. *J. Med. Genet.*, **33**, 1–4.
- Thinakaran, G., Borchelt, D.R., Doan, A., Slunt, H.H., Lee, M.K., Nordstedt, C., Seeger, M., Gandy, S.E., Hardy, J.A., Levey, A.I., Price, D.L., and Sisodia, S.S. (1996) Endoproteolytic processing and protein topology of presenilin 1. *Society for Neuroscience*, **22**, 728.
- Thinkaran, G., Borchelt, D.R., Lee, M.K., Slunt, H.H., Spitzer, L., Kim, G., Ratovisky, T., Davenport, F., Nordstedt, C., Seeger, M., Levey, A.I., Gandy, S.E., Jenkins, N.A., Copeland, N., Price, D.L., and Sisodia, S.S. (1996) Endoproteolysis of Presenilin 1 and accumulation of processed derivatives *in vivo*. *Neuron*, **17**, 181–190.

- Uchihara, T., El Hachimi, H.K., Duyckearts, C., Foncin, C., Fraser, P.E., Levesque, L., St George-Hyslop, P.H., and Hauw, J.J. (1996) Widespread immunoreactivity of presenilin in neurons of normal and alzheimers disease brain: double labeling immunohistochemical study. *Acta Neuropathologica*, **92**, 325–330.
- Valdes, J.M., Tagle, D.A., and Collins, F.S. (1994) Island Rescue PCR: a rapid and efficient method for isolating transcribed sequences from YACs and cosmid. *Proc. Natl Acad. Sci. USA*, **91**, 5377–5381.
- Van Broeckhoven, C. (1995) Presenilins and Alzheimer Disease. *Nature Genet.*, **11**, 230–232.
- Van Broeckhoven, C., Backhovens, H., Cruts, M., De Winter, G., Bruylant, M., Cras, P., and Martin, J.-J. (1992) Mapping of a gene predisposing to early-onset Alzheimer's Disease to chromosome 14q24.3. *Nature Genetics*, **2**, 335–339.
- van Broeckhoven, C., Backhovens, H., Cruts, M., Martin, J.J., Crook, R., Houlden, H., and Hardy, J. (1994) APOE genotype does not modulate age of onset in families with chromosome 14 encoded Alzheimer's Disease. *Neurosci. Lett.*, **169**, 179–180.
- Van Duijn, C.M., de Knijff, P., Crutts, M., Wehnert, A., Havekes, L.M., Hofman, A., and Van Broeckhoven, C. (1994) Apolipoprotein ε4 allele in a population based study of early onset Alzheimer Disease. *Nature Genet.*, **7**, 74–78.
- Vito, P., Lacana, E., and D'Adamio, L. (1996) Interfering with apoptosis: Ca(2+)-binding protein ALG-2 and Alzheimer's disease gene ALG-3. *Science*, **271**, 521–525.
- Walter, J., Capell, A., Grunberg, J., Pesold, B., Schindzielorz, A., Prior, R., Podlisny, M.B., Fraser, P., St George-Hyslop, P.H., Selkoe, D., and Haass, C. (1996) The Alzheimer's Disease associated Presenilins are differentially phosphorylated proteins located predominantly within the endoplasmic reticulum. *Molec. Medicine*, **2**, 673–691.
- Wisniewski, H.M., and Rabe, A. (1986) Discrepancy between Alzheimer type neuropathology and dementia in person with Downs syndrome. *Ann. N.Y. Acad. Sci.*, **477**, 247–260.
- Wisniewski, K.E., Dalton, A.J., McLachlan, D.R.C., Wen, G.Y., and Wisniewski, H.M. (1985) Alzheimer's disease in Down's syndrome: clinico-pathologic studies. *Neurology*, **17**, 278–282.
- Wisniewski, T., and Frangione, B. (1992) Apolipoprotein E: a pathological chaperone protein in patients with cerebral and systemic amyloid. *Neurosci. Letts.*, **135**, 235–238.
- Wisniewski, T., Lalowski, M., Golabek, A., Vogel, T., and Frangione, B. (1995) Is Alzheimer's disease an ApoE amyloidosis? *Lancet*, **345**, 956–958.
- Wisniewski, T., Palha, J.A., Ghiso, J., and Frangione, B. (1996) S182 protein in Alzheimer's neuritic plaques. *Lancet*, **346**, 1366–1366.
- Wolozin, B., Iwasaki, K., Vito, P., Ganjei, K., Lacana, E., Sunderland, T., Zhao, B., Kusiak, J.W., and D'Adamio, L. (1996) Participation of Presenilin 2 in apoptosis: enhanced basal activity conferred by an Alzheimer Mutation. *Science*, **274**, 1710–1713.
- Wong, L., Liang, Y., Tsuda, T., Fong, Q., Galway, G., Alexandrova, N., Rogeava, E., Lukiw, W., Smith, J., Rogaev, E., Crapper MacLachlan, D., and St George-Hyslop, P. (1993) Mutation of the gene for the human lysosomal serine protease Cathepsin G is not the cause of aberrant APP processing in Familial Alzheimer Disease. *Neurosci. Lett.*, **152**, 96–98.
- Wong, P.C., Zheng, H., Chen, H., Trumbauer, M.E., Roskams, A.J., Chen, H.Y., Van der Ploeg, L.H., Price, D.L., and Sisodia, S.S. (1996) Functions of the presenilins: generation and characterization of presenilin-1 null mice. *Society for Neuroscience*, **22**, 728.
- Wragg, M., Hutton, M., Talbot, C., Busfield, E., Han, S.W., Lendon, C., Clark RF, Morris, J.C., Goate, A., Hardy, J., Rossor, M., Houlden, H., Karran, E., Roberts, G., and Craddock, N. (1996) Genetic association between and intronic polymorphism in presenilin 1 gene and late onset Alzheimer Disease . *Lancet*, **347**, 509–512.

- Yamatsuji, T., and Nishimoto, I. (1996), G., protein-mediated neuronal DNA fragmentation induced by familial Alzheimer's disease associated mutants of APP. *Science*, **272**, 1349–1352.
- Yankner, B.A. (1996) Mechanisms of neuronal degeneration in Alzheimer's disease. *Neuron*, **16**, 921–932.
- Yankner, B.A., Duffy, L.K., and Kirschner, D.A. (1990) Neurotrophic and neurotoxic effects of amyloid β protein: reversal by tachykinin neuropeptides. *Science*, **250**, 279–282.
- Zheng, H., Jiang, M., Trumbauer, M.E., Hopkins, R., Sirinathsinghji, D.J., Stevens, K.A., Corner, M.W., Slunt, H.H., Sisodia, S.S., Chen, H.Y., and Van der Ploeg, L.H. (1996) Mice deficient for the amyloid precursor protein gene. *Ann. N.Y. Acad. Sci.*, **777**, 421–426.

**MOLECULAR AND CELLULAR BIOLOGY OF THE
β-AMYLOID PRECURSOR PROTEIN AND
AMYLOID β-PEPTIDE**

4. THE BIOLOGICAL ACTIVITIES AND FUNCTION OF THE AMYLOID PRECURSOR PROTEIN OF ALZHEIMER'S DISEASE

GERD MULTHAUP¹, COLIN L. MASTERS², KONRAD BEYREUTHER¹ and
ROBERTO CAPPAL²

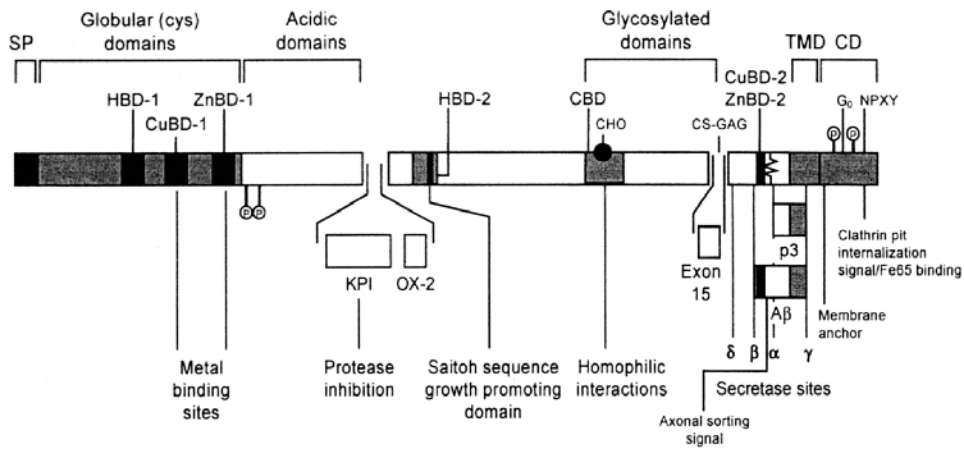
¹*ZMBH-Center of Molecular Biology, University of Heidelberg, Im Neuenheimer Feld
282, D-69120 Heidelberg, Germany*

²*Department of Pathology, The University of Melbourne and the Mental Health Research
Institute of Victoria, Parkville Victoria, 3052, Australia*

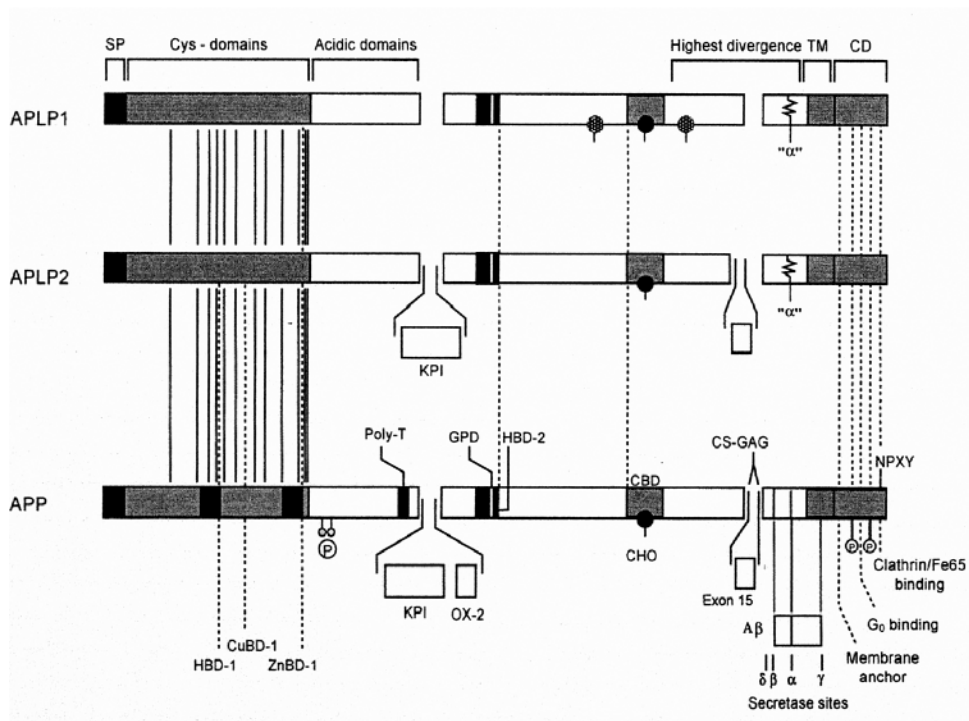
INTRODUCTION

We start from the position that the APP molecule (and related members of the APP superfamily) will have many different activities, but for each APP gene product there will be only one primary function. Moreover, this function will be the same in neuronal and non-neuronal cells. This paper summarizes a number of activities, but it has to be admitted at the outset that the primary function of APP remains unknown. The structural map of APP and the APLP1 and 2 orthologs is shown in the Figure, and a summary of reported activities and corresponding proposed functions is presented in the table. The phylogenetic relationships of the APP superfamily has been presented elsewhere (Coulson *et al.*, manuscript submitted).

From the high degree of evolutionary conservation of the endo- and ectodomains of APP and its widespread tissue expression, APP has been implicated in a variety of cellular processes and events. Secreted isoforms of APP (sAPP) containing a Kunitz-type protease inhibitor (KPI) consensus sequence have a role in regulation of extracellular protease activity (Oltersdorf *et al.*, 1989) and as inhibitors of proteases involved in the regulation of the coagulation cascade (Smith *et al.*, 1990; Van Nostrand *et al.*, 1990) (see below). The carboxy terminus of sAPP has been shown to be involved in regulating intracellular calcium levels and thus exerting neuroprotective activities (Mattson *et al.*, 1993) (see below). Another possible function of the ectodomain of secreted or membrane-associated forms of APP is as regulator of neuronal-cell or cell-matrix interactions, cell growth and synaptic plasticity (Milward *et al.*, 1992; Mucke *et al.*, 1994; Roch *et al.*, 1992; Saitoh *et al.*, 1989; Schubert *et al.*, 1989). The ability of APP to stimulate cell adhesion and growth does not depend on the KPI domain but may derive from the high affinity of APP for heparin, heparan sulfate proteoglycans (HSPG), laminin and collagen type IV (Beher *et al.*, 1996; Breen, 1992; Klier *et al.*, 1990; Multhaup, 1994; Multhaup *et al.*, 1994; Schubert *et al.*, 1989; Small *et al.*, 1994). Specific APP species have been detected as a result of alternative exon splicing. For example, a chondroitin sulfate glycosaminoglycan (CS GAG) modification of L-APP and L-APLP2 isoforms which lack 18 amino acids encoded by exon



(A)



(B)

Figure 1.

Table 1. Summary of activities and corresponding proposed function of APP.

<i>Observed Activity</i>	<i>Proposed Function</i>	<i>References*</i>
Binding and reducing copper. Binding to zinc.	Metal ion homeostasis.	1
Binding to HSPG, collagen, glypican and laminin. Stimulation of cell-extracellular matrix adhesion.	Regulation of neurite outgrowth. Mediator of cell-cell and cell-matrix interactions.	2
Stimulation of neurite outgrowth and synaptogenesis.	Regulation of neurite outgrowth and/or synaptic plasticity.	3
Stimulation of mitogenesis, MAP kinases and G _o proteins.	Regulation of cell proliferation, differentiation and survival.	4
Regulation of calcium homeostasis, cGMP levels and K ⁺ channel activation. Protection against excitotoxicity, hypoglycemia and brain ischemia.	Neuroprotection.	5
Serine protease inhibition.	Regulation of blood coagulation.	6

*1. Hesse *et al.*, 1994, Multhaup *et al.*, 1996, Bush *et al.*, 1993.

2. Behr *et al.*, 1996, Breen 1992, Williamson *et al.*, 1996, Multhaup, 1994, Klier *et al.*, 1990, Small *et al.*, 1994, Narindrasorasak *et al.*, 1992, Schubert *et al.*, 1989, Schubert *et al.*, 1989.

3. Milward *et al.*, 1992, Mucke *et al.*, 1994, Huber *et al.*, 1993, Doyle *et al.*, 1990.

4. Greenberg *et al.*, 1995, Saitoh *et al.*, 1989, Schubert *et al.*, 1989, Ninomiya *et al.*, 1993, Okamoto *et al.*, 1996.

5. Mattson *et al.*, 1993, Furukawa *et al.*, 1996, Schubert and Behl, 1993, Barger *et al.*, 1995, Smith-Swintosky *et al.*, 1994, Ishida *et al.*, 1997.

6. Smith *et al.*, 1990, Van Nostrand *et al.*, 1990.

15 of APP convert these proteins to chondroitin sulfate proteoglycans (CSPGs) that are produced by astrocytes (Shioi *et al.*, 1995) and stimulate the adhesion of both glia and neuronal cells (Wu *et al.*, 1997).

APP, ZINC AND COPPER

Studies on metal-ion binding to APP were performed to investigate an association between metals and APP metabolism. A novel Zn(II) binding motif was discovered in the cysteine rich N-terminal region of APP between residues 181–200 (Bush *et al.*, 1993) and is distinct from the Zn(II) binding sites in the A β region (Bush *et al.*, 1994). The zinc binding site maps to exon 5 and is present in all members of the APP superfamily. Zinc (II) binding has been shown to modulate the functional properties of APP, possibly by enhancing its macromolecular conformation. Incubation of APP with Zn(II) increases binding of APP to heparin (Multhaup, 1994; Multhaup *et al.*, 1994) and it potentiates the inhibition of coagulation factor XIa by APP-KPI⁺ isoforms (Komiyama *et al.*, 1992; Van Nostrand, 1995). Zn(II)

binding also influences the cleavage of the APP molecule, potentially via alpha-secretase activity (Bush *et al.*, 1994; Li *et al.*, 1995). The *in vivo* influences of zinc on APP metabolism were tested in rats given a dietary Zn(II) supply. This caused an increase in membrane associated forms and a reduction of soluble forms of APP (Whyte *et al.*, 1997). This may be due either to altered APP trafficking or processing. An active transport of neuronal Zn(II) along cell processes or from cell to cell has been hypothesized (Simons *et al.*, 1995).

Whereas Zn(II) is assumed to play a purely structural role, we found that APP binds Cu(II) with a dissociation constant of 10nM at pH 7.5 and reduces it to Cu(I) (Hesse *et al.*, 1994; Multhaup *et al.*, 1996). Since Zn(II) exists exclusively in one oxidation state, only APP/Cu(II) complexes are sensitive to redox reactions. The APP copper binding motif corresponds to type II copper binding proteins and is encompassed by residues APP135–155 within exon 4. The reduction of Cu(II) to Cu(I) by APP results in a corresponding oxidation of cysteines 144 and 158 in APP (Multhaup *et al.*, 1998, *Biochemistry*, in press) that involves an intramolecular reaction leading to a new disulfide bridge. This reaction was found to be very specific, as APP did not bind and reduce other metals such as Fe(III), Ni (II), Co(II) or Mg(II). Thus, Cu(II) binding leads to oxidative modification of APP, resulting in cystine and Cu(I) formation which indicates APP has an *in vitro* function in electron transfer to Cu(II).

APP, COPPER TRANSPORT AND NEURONAL DEGENERATION

Copper is an essential trace element in the brain that is required for a number of enzyme activities including cytochrome *C* oxidase, Cu/Zn-superoxide dismutase (Cu/Zn-SOD) and dopamine- β -hydroxylase (a key player in the catecholamine biosynthetic pathway in the nervous system) (Linder and Hazegh Azam, 1996; Stewart and Klinman, 1988). Zinc and copper ions are needed for thermally stable native Cu/Zn-SOD and to restore full catalytic activity. Whereas zinc can be substituted with cadmium, mercury and cobalt ions (Beem *et al.*, 1974), no metal can replace copper in restoring catalytic function.

Despite the essential role in electron transfer, copper is highly reactive and potentially toxic. Thus specialized pathways have evolved for the trafficking of this metal within cells (Hung *et al.*, 1997). The following data provide strong evidence that APP has a role in copper transport. In neurons, APP is first delivered from the cell body to the axonal surface and then to the dendritic plasma membrane (Simons *et al.*, 1995; Yamazaki *et al.*, 1995). The function of APP transport in epithelial and neuronal cells is not known, but we assume that APP has an important physiological role in the cellular transport of the metal ions Zn(II) and Cu(II)/(I) both in the periphery and in the central nervous system. Indirect evidence indicates that APP may also function in copper uptake during internalization. APP molecules are normally reinternalized from the cell surface via endocytosis signals in the cytoplasmic tail (Haass *et al.*, 1992; Koo and Squazzo, 1994) and then processed to A β . The safe sequestration of transition metal ions is probably an important antioxidant defense in its own right. This is compatible with the presence of a single Cu(II)/Cu(I) binding site in APP (Multhaup *et al.*, 1996) and the fact that organisms go to considerable energetic expense in the handling of transition metal ions to minimize availability and hence reactivity.

APP mediated reduction of bound Cu(II) to Cu(I) suggests APP is acting as an extra-cellular copper reductase. The finding that an ion-mediated redox reaction leads to disulfide bridge formation in APP

would indicate that free sulfhydryl groups in APP are involved (Multhaup *et al.*, 1996). This is compatible with our knowledge on copper uptake in eucaryotic cells since inhibition of copper transport by cuprous chelators or stimulation of copper uptake by ascorbate suggests that copper is taken up as Cu(I) rather than Cu(II). Furthermore, sulfhydryl-modifying reagents inhibit the uptake of copper, implicating that sulfur amino acids are required for the transport process *in vivo* (Harris, 1991; Percival and Harris, 1989). A regulatory mechanism for copper uptake has been discovered in the copper-dependent turnover of Ctrlp protein (Ooi *et al.*, 1996). When cells are grown in low concentrations of copper, Ctrlp is a stable protein. But an increase in copper concentrations up to 10 μ M induces cleavage of Ctrlp at the cell surface (Ooi *et al.*, 1996). This cleavage is believed to release the extracellular domain of Ctrlp that contains the copper binding site and thus inhibits further copper uptake. This may represent a general mechanism for other plasma proteins to regulate the uptake of ligands. This would also be comparable with the alpha-secretase cleavage of APP which occurs on or close to the cell surface (Sisodia *et al.*, 1990) to release a large amino-terminal fragment of APP and precludes formation of full length A β . Such a proteolytic pathway may inhibit copper uptake since the amino-terminal domain of APP is extracellular and binds copper. This alternative and non-amyloidogenic pathway indicates that APP could be a "mammalian Ctrlp" and may be a rate-limiting component responsible for the delivery of copper to the cytosol of mammalian cells. In contrast, if cells are grown in low concentrations of copper the internalization of cell surface APP could be favored and the intracellular processing would be shifted to β - and γ -secretase cleavage of APP, thereby increasing the production of the A β amyloid protein of Alzheimer's disease.

Two human inherited disorders illustrate the importance of copper in recessive disorders of copper metabolism, Wilson disease and Menkes disease. The defective genes encode membrane Cu-transporting P-type ATPases. A failure to express functional proteins can result in decreased copper efflux, whilst overexpression of the Menkes gene will increase copper efflux and confer a stronger resistance to the toxic effects of copper (Camakaris *et al.*, 1995). Neuronal degeneration is a discrete clinical finding of Menkes disease and also of familial amyotrophic lateral sclerosis (FALS). In FALS point mutations in Cu/Zn-superoxide dismutase (Cu/Zn-SOD) exhibit enhanced free radical-generating activity, while its dismutation activity is identical to that of the wild-type enzyme (Yim *et al.*, 1996). Currently, much evidence argues strongly that the disease arises not from loss of SOD function but rather from an adverse or toxic gain of function of the mutant SOD1 molecule (Brown, 1995; Wong *et al.*, 1995). Transgenic mice that overexpress the human SOD1 gene develop a clinical form of motor neuron disease analogous to human FALS (Gurney *et al.*, 1994). An insight into the mechanism has been gained with the discovery that SOD is inactivated by H₂O₂ which rapidly reduces Cu(II) at the active site (Hodgson and Fridovich, 1975; Sato *et al.*, 1992) and that Cu/Zn-SOD was found to generate free $\cdot$ OH radicals from H₂O₂ (Yim *et al.*, 1996). In an *in vitro* system, FALS-associated mutant SOD1 enzyme catalyzes the reduction of H₂O₂, thereby acting as a peroxidase (Wiedau-Pazos *et al.*, 1996). Thus, Cu/Zn-SOD has a peroxidative function that utilizes its own dismutation product, H₂O₂, as a substrate. This was found to occur more rapidly with mutant than wild-type SOD1, with the mutants at least twice as reactive as the wild-type enzyme. Thus, the strong association found between familial amyotrophic lateral sclerosis (FALS) and mutations in the Cu/Zn-SOD gene is the most convincing evidence so far for a link between neurological disorders and oxygen radical formation, implying that oxygen radicals might be responsible for the selective degeneration of motor

neurons occurring in FALS (Wiedau-Pazos *et al.*, 1996; Yim *et al.*, 1997; Yim *et al.*, 1996). SOD mutations adversely affect Cu homeostasis, thus leading to dysfunction and death of neurons.

In Alzheimer's disease, APP-Cu(I) complexes on the surface of neurons may be particularly vulnerable to peroxides generated by extracellular forms of SOD. Such complexes are spontaneously formed since APP itself reduces bound Cu(II) to Cu(I). This Cu(II) ion-mediated redox reaction leads to disulfide formation in APP and to the formation of APP-Cu(I) complexes, even in the absence of hydrogen peroxide (Multhaup *et al.*, 1996). Sporadic Alzheimer's disease could arise from a perturbation of free radical homeostasis and resulting neuronal toxicity by reactive oxygen species. This model is consistent with the slow onset of AD: younger persons may have greater antioxidant capacity and can withstand free radical stress. Aging coupled to environmental insults or genetic defects could exacerbate the consequences of APP fragmentation. The most prominent risk factor associated with late onset AD has been shown to be linked to cytotoxicity modulated by the varying antioxidant activity of the apoE isoforms (Miyata and Smith, 1996). The $\epsilon 4$ allele has a higher frequency in AD patients than in age matched controls and was found to possess the lowest activity in protecting cells from hydrogen peroxide cytotoxicity (Miyata and Smith, 1996). Thus, the hypothesis of radical-based APP fragmentation and neurotoxicity of degradation products (Kozlowski *et al.*, 1992) presents several novel therapeutic strategies for all forms of AD.

AMYLOID A β AND METAL-ION BINDING

The A β peptide can bind Zn(II) in a specific and saturable manner (Bush *et al.*, 1994). Zn(II) binding caused soluble A β to precipitate at physiological pH and salt concentrations *in vitro*. Concentrations of Zn(II) above 300nM rapidly destabilized human A β 1–40 solutions, inducing amyloid formation with properties similar to that present in the cerebral cortex of AD patients. Human A β 1–40 contains a low and a high affinity binding site localised to A β residues 6–28. In contrast to human A β , the rat A β 1–40 remains soluble in the presence of up to 10 μ M Zn(II). This would suggest the sequence differences at positions 5, 10 and 13 alter the physicochemical properties of the rat peptide. The rat A β peptide had Zn binding properties consistent with only a low affinity binding site being present (Bush *et al.*, 1994). In addition, the precipitating effect of Zn(II) on A β 1–40 aggregation could be increased by decreasing the pH within a physiological range (Atwood *et al.*, 1997). *In vivo*, mildly acidic conditions may unmask the second metal binding site in the human sequence to release the metal ions by protonation of the histidine residues and thus promote the aggregation of the peptide. Cu(II) has also been shown to bind to A β and may stabilize the dimeric forms of A β . It was found that decreasing the pH caused an increasing in Cu(II) mediated precipitation of A β from solution or suspension (Atwood *et al.*, 1997).

Free radical catalysts such as Fe(III) and Al(III) could markedly accelerate the *in vitro* aggregation of radioiodinated human A β into amyloid (Mantyh *et al.*, 1993). Dyrks *et al.* reported the transformation of larger amyloidogenic APP-fragments, such as A4CT, into highly insoluble stable aggregates by metal catalyzed oxidation (Dyrks *et al.*, 1992). The transformation of soluble A β and A4CT into insoluble and aggregating molecules occurred after hydrogen peroxide was added in conjunction with metal catalyzed oxidation systems such as hemoglobin and hemin. The aggregation process was prevented in the presence of radical scavengers or free amino acids. It was concluded that aggregation was induced by amino acid oxidation and protein cross-linking.

HEPARIN BINDING IN THE FUNCTION OF APP

Two putative heparin binding domains in APP have been reported (Multhaup, 1994, Small *et al.*, 1992) although a varying number of candidate heparin-binding sequences (up to four) were suggested when simple cation-exchange chromatography was exploited to define "heparin-binding" (Clarris *et al.*, 1997). Such interactions are applied to the purification of negatively charged proteins (at neutral pH), which are adsorbed to immobilized heparin at low ionic strength and subsequently eluted with salt. Thus, a low affinity heparin binding site was located within residues 96–110 of APP (Small *et al.*, 1994). This sequence, including the low affinity heparin binding site, is poorly conserved within the APP superfamily and binds three orders of magnitude less tightly to heparin *in vitro*. The high affinity heparin binding site, which interacts with immobilized heparin with affinity rather than cation exchange chromatography, was a basic region of 22 amino acids within the carbohydrate domain of APP (aa 316–337 in APP₆₉₅) and has homology to the heparin binding site of NCAM (Multhaup, 1994). The apparent dissociation constant derived from Scatchard transformation could be determined to be 0.3 nM for APP-binding to heparin Sepharose. This high affinity heparin binding site is conserved between human, mouse and rat APP and shares strong homology with human, mouse and rat APLP2 (Kang *et al.*, 1987, Sandbrink *et al.*, 1994, Shivers *et al.*, 1988, Sprecher *et al.*, 1993, Vidal *et al.*, 1992, Yamada *et al.*, 1987) (and see the Figure). The sequence of mouse APLP1 also contains such a sequence within residues 313–334 (Wasco *et al.*, 1992) and in fact both orthologs of APP were found to bind heparin (Bush *et al.*, 1994). The domain is partially conserved in the Drosophila homologue APPL within residues 411–432 (Rosen *et al.*, 1989). CD studies showed that this high affinity heparin binding site also has alpha-helical characteristics (Clarris *et al.*, 1997) which may predispose it to bind heparin in combination with a cluster of basic residues (Margalit *et al.*, 1993). Recently, Scatchard analysis of the glypican binding to APP indicated one class of binding sites with an equilibrium constant of 2.8 nM which is fully in agreement with the high affinity heparin binding site encoded by exons 9 and 10 of APP (Williamson *et al.*, 1996). Thus, APP binding to heparin *in vitro* also suggests that APP-induced neurite outgrowth (Milward *et al.*, 1992; Roch *et al.*, 1992) may be mediated through the high affinity heparin binding site *in vivo*, as suggested earlier (Multhaup, 1994). Recent studies have provided more evidence for this suggestion when heparin-like molecules, such as the HSPGs glypican and perlecan were found to compete with endogenous proteoglycans for the neurite outgrowth-promoting activity of APP (Williamson *et al.*, 1996).

Heparin and heparan sulfate, the latter generally less sulfated than heparin, are glycosaminoglycans that are involved in biological processes such as cytokine action, cell-adhesion, and regulation of enzymic catalysis. Heparin is believed to act *in vivo* as a specific regulator of the structure and function of basement membranes (Yurchenco *et al.*, 1990). Local variation in the concentration of heparin-like macromolecules was suspected to be crucial for the interaction of APP and collagens (Beher *et al.*, 1996). APP can interact with the $\alpha 1$ (I)CB6 fragment of collagen types I and IV and this interaction can be influenced by glycosaminoglycans. This interaction is regulated by heparin, suggesting both APP and heparin probably share the same binding site on collagen. The regulation of APP binding to collagen type I by heparin reflects competitive binding between APP and collagen for heparin since both components can interact with glycosaminoglycans (Beher *et al.*, 1996; Cole and Glaser, 1986; Lindahl and Höök, 1978).

The high affinity zinc(II) binding site has been suggested to modulate heparin binding of APP (Bush *et al.*, 1993) because zinc(II) was found to increase the amount of total binding of APP to heparin Sepharose (Bush *et al.*, 1993). To explore the significance of this finding in more detail, we studied the affinity of APP binding to heparin at increasing zinc(II) concentrations by surface plasmon resonance (SPR) using biosensor-based technology. This method has the potential to analyze quantitatively binding of ternary complexes such as binding of APP to heparin in the presence of zinc(II) at high sensitivity. We found that zinc(II) strengthened the binding of APP to heparin. Zinc(II) is known to occur in vesicles at concentrations of up to 300 μ M in zinc(II)-sensitive mossy fibres of the hippocampus and to be released during neuronal activity (Margalit *et al.*, 1993). Zinc(II) is therefore in principle capable of modulating the interaction of APP with the heparan sulfate moiety of proteoglycans. The fact that zinc(II) strengthens APP binding to heparin demonstrates an allosteric interaction between the residues encoded by exons 5 (zinc(II) binding site) and 9 and 10 (heparin binding site). In addition, the evolutionary conservation of the zinc(II) binding site in APP down to the level of *C. elegans* (Daigle and Li, 1993) indicates that the function of this domain may be critical to the physiological role of the members of the APP superfamily. Zinc(II) binding may also influence APP processing since in the presence of proteases such as trypsin the stability of APP enriched by heparin affinity chromatography from plasma of patients with AD was greatly reduced at high concentrations of zinc (Bush *et al.*, 1993). Thus, the functional zinc(II) binding site of APP may be of great importance for AD pathogenesis because abnormal zinc metabolism in AD and Down's syndrome has been reported (Franceschi *et al.*, 1988, Napolitano *et al.*, 1990). A disturbed homeostasis of extracellular zinc(II) in AD (Bush *et al.*, 1993) may interfere with the normal binding of APP to HSPG (Narindrasorasak *et al.*, 1992). The evidence for the involvement of HSPG, such as perlecan, in amyloid formation is provided by its presence in amyloid plaques (Snow *et al.*, 1995). Heparin and heparan sulfate may play a role in the early stages of neurofibrillary tangle formation (Goedert *et al.*, 1996) and also induce extracellular amyloid fibril formation *in vitro* (Fraser *et al.*, 1992).

THE APP MOLECULE AS A REGULATOR OF HEMOSTASIS

The KPI domain represents one of the best understood regions of the APP molecule both in terms of function and structure. The KPI containing APP isoforms (APP-KPI⁺) corresponded to the previously described protease nexin II molecule (Oltersdorf *et al.*, 1989, Van Nostrand *et al.*, 1989). Protease nexins are a widely expressed gene family of serine protease inhibitors secreted by a variety of cells (Knauer *et al.*, 1983). Protease nexin II was originally identified as an inhibitor of the epidermal growth factor binding protein (a serine esterase), γ -subunit of nerve growth factor and trypsin (Knauer and Cunningham, 1982, Van Nostrand and Cunningham, 1987). APP-KPI⁺ is present in high amounts in the platelet α -granules where it is released upon platelet activation. The Kunitz-type inhibitor protein corresponds to a low molecular serine protease inhibitor present in virtually all cell types (Fioretti *et al.*, 1983). The KPI sequence has been incorporated in a variety of proteins ranging from APP to chicken collagen VI (Ikeo *et al.*, 1992). The differences in substrate specificity between the different KPI sequences suggest a diversity of activities between the various family members. It has been calculated that the APP gene acquired the KPI sequence from evolutionary precursors by exon shuffling about 270 million years ago (Ikeo *et al.*, 1992).

Numerous reports have confirmed the activity of the APP-KPI domain as a bona fide serine protease inhibitor. The majority of these studies have analyzed the ability of APP-KPI⁺ to inhibit proteases of the coagulation cascade. The coagulation cascade is activated following vascular injury and involves a complex series of transformations of proenzymes to activated enzymes. Two major pathways exist, the intrinsic and the extrinsic (tissue) pathway. The intrinsic pathway is activated following contact of factor XII with an abnormal surface. This leads to activation of factor XII (termed factor XIIa) and the sequential interaction and activation of factors XI, IX, VIII and X. The end result is the formation of thrombin which converts soluble fibrinogen to insoluble fibrin. The demonstration that APP-KPI⁺ can inhibit different coagulation factors relates not only to the KPI region and coagulation but has provided important insights into how other parts of the APP molecule interact to regulate its function.

Native APP-KPI⁺ isoforms inhibit factor XIa activity with a $K_i=450$ pM (Smith *et al.*, 1990). In contrast, APP-KPI⁺ inhibits trypsin with a $K_i=20$ pM. The stoichiometry of APP to factor XIa binding was 2:1 whilst the ratio with trypsin was 1:1 (Scandura *et al.*, 1997, Van Nostrand, 1995). Recent studies have defined the interaction with factor XIa as reversible slow tight-binding which is typical to other Kunitz-type inhibitors (Smith *et al.*, 1990; Van Nostrand *et al.*, 1990). Mutagenesis studies have started to delineate the critical residues involved and have shown that a lysine for arginine substitution in the KPI reactive center causes a 25 fold decrease in APP-KPI inhibitory activity on factor XIa, but not on trypsin, chymotrypsin, factor IXa and factor Xa (Van Nostrand *et al.*, 1995). In contrast, the mutation increased the ability of APP-KPI⁺ to inhibit plasmin (a fibrinolytic enzyme which degrades different clotting factors including fibrin). These findings indicate particular residues could alter factor specificity and lead to the proposed design of KPI-based anti-coagulants which target specific factors.

As noted above, the APP molecule has binding sequences for heparin and zinc. Inhibition of factor XIa by APP is modulated by the addition of heparin (Smith *et al.*, 1990). A five-fold increase in inhibition occurs with the addition of > 1 μ M heparin. In contrast, heparin does not alter APP-KPI⁺ inhibition of trypsin. The APP zinc binding site can also modulate APP inhibitory activity. The addition of Zn (II) ions (> 1 μ M) result in a three-fold increase in inhibition (Van Nostrand, 1995). Zinc ions have no effect on trypsin or chymotrypsin inhibition by APP-KPI⁺. The zinc effect is presumably mediated by the zinc binding sequence of APP since the activity of a recombinant fragment encompassing the KPI domain alone was not altered by zinc. Zinc and heparin can act synergistically and cause a modest (50%) increase in APP inhibitory activity (Van Nostrand, 1995). This is consistent with studies showing that zinc increases heparin binding to APP (Bush *et al.*, 1993). However, it is also possible that zinc and heparin are binding directly to factor XIa and are altering its activity and/or binding to APP. Studies with zinc and heparin APP mutants would demonstrate the direct involvement of APP zinc and heparin binding sites.

When circulating in plasma, Factor XIa is normally bound to high molecular weight kininogen (HMK). HMK can protect factor XIa from APP-KPI⁺ mediated inhibition by increasing the K_i twofold (Scandura *et al.*, 1997). However, in the presence of Zn(II) ions the protective effects of HMK are abolished. The existence of zinc binding sites on both HMK and APP lead to the intriguing hypothesis that factor XIa activity is modulated by the absence (HMK is active) or presence (APP-KPI⁺ is active) of zinc. When assayed in activated platelet releasates, factor XIa activity decreases over time, which is consistent with the release of APP from the platelet alpha granules after platelet activation (Van Nostrand *et al.*, 1990). Interestingly, the protective effects of HMK predominate over zinc ions when

analysed in the presence of activated platelet releasates. It is known that zinc and HMK are necessary for factor XIa to bind to activated platelets. It was therefore proposed that HMK and zinc ions promote the binding of factor XIa to the platelet surface and in this environment it is protected from APP-KPI⁺ mediated inactivation. Whilst the exact mechanism needs to be determined, the data indicate that these APP binding molecules strongly influence the function of APP in coagulation.

Coagulation factor IXa is part of the intrinsic system and after binding to other plasma molecules activates factor X. Deficiencies in factor IXa results in spontaneous bleeding and in some cases intracerebral hemorrhage. Both a recombinant KPI domain (Wagner *et al.*, 1992) and purified native APP cause a two-fold inhibition in factor IXa coagulation activity (Schmaier *et al.*, 1993). Gel filtration experiments show that factor IXa and APP-KPI⁺ form a complex with a 1:1 stoichiometry. Inhibition of factor XIa is also modulated by the addition of heparin which causes a twofold decrease in the K_i value to 40 pM. Importantly, the K_i for the recombinant KPI domain is three orders of magnitude higher than full length APP, indicating regions other than the KPI are necessary for maximal inhibition. Recently, the low density lipoprotein receptor-related protein (LRP) has been shown to be a receptor for either soluble (Kounnas *et al.*, 1995) or cell-associated (Knauer *et al.*, 1996) APP-KPI⁺ containing isoforms. LRP is a cell surface molecule which binds many different ligands and results in their subsequent catabolism (Kounnas *et al.*, 1995). This interaction could be particularly relevant since APP complexed with factor IXa is catabolised in an LRP-dependent manner, suggesting LRP may be involved in the clearance of APP-KPI⁺-proteinase complexes. Determining the uptake and catabolism of APP-KPI complexed with other coagulation factors would be of interest.

Coagulation factor Xa forms part of the prothrombinase complex that results in prothrombin being cleaved to the active thrombin species. APP can inhibit factor Xa with a K_i of 20nM (Mahdi *et al.*, 1995). In contrast to its inhibition-enhancing effects on other coagulation factors, the addition of heparin had the opposite effect and prevents APP-KPI⁺ mediated inhibition of factor Xa. Interestingly, APP is itself a substrate for factor Xa cleavage and is cleaved within the amino-terminal heparin binding region after arginine 102. Disruption of the heparin binding domain may allow modulation of APP function by preventing its association with heparin (Mahdi *et al.*, 1995). Another example of coagulation factor processing of APP is described for factor XIa cleaving within the middle of the RHDS sequence of the A β sequence in APP695 (Saporito-Irwin and Van Nostrand, 1995). The RHDS sequence has been linked to the cell adhesion promoting activity of APP (Ghisso *et al.*, 1992); factor XIa treatment reduces the cell adhesive properties of APP and A β (Saporito-Irwin and Van Nostrand, 1995). This presents a complex relationship between APP and the coagulation factors, with competing regulation of the activities of each component.

Discussions on the function of APP need to consider the other members of the APP gene family of which APLP2 is the only ortholog to contain a KPI domain (Slunt *et al.*, 1994, Sprecher *et al.*, 1993, Wasco *et al.*, 1992). A recombinant fragment encompassing the APLP2-KPI domain inhibits the coagulation factors XIa and IXa (Van Nostrand *et al.*, 1994). The APLP2-KPI domain displays similar inhibition constants against purified factor IXa, trypsin and chymotrypsin activity in comparison to APP-KPI. However, the APLP2-KPI domain is 20-fold less effective in inhibiting factor XIa activity than the APP-KPI domain. A reduction in activity is also seen when measuring APLP2-KPI activity in plasma, suggesting APLP2-KPI may have an as yet unidentified target. This data indicates that the

APP gene family constitutes a group of proteins capable of regulating components of the coagulation pathway.

How do the above studies relate to a possible *in vivo* function for APP-KPI⁺ in hemostasis? In comparison to antithrombin III (the presumed plasma inhibitor of factors IXa and Xa), APP-KPI⁺ is at least 70-fold more active in inhibiting factor IXa and equipotent in inhibiting factor Xa. However, based on plasma concentrations, antithrombin in (at 4 μ M) would be the major inhibitor when compared to concentrations of APP (approximately 1 nM) (Scandura *et al.*, 1997). The low plasma levels of APP suggest a model where the main site of action of APP is as an inhibitor on cell membranes (endothelial cell and platelet surfaces). To support this, it is known high levels of APP expression occur in the platelet α -granules and membrane surface (Li *et al.*, 1994, Smith *et al.*, 1990, Van Nostrand *et al.*, 1990). Following platelet activation, α -granule APP is released and full-length APP is proteolytically processed to release sAPP and a membrane bound form (Li *et al.*, 1995). The overall result is a three-fold increase in surface APP detectability. As a result of vascular injury, platelets would be activated and significant amounts of APP may therefore accumulate to high concentrations in the immediate microenvironment of the platelet. It is interesting to note that heparin can modulate the inhibitory activity of antithrombin III (Rosenberg, 1985). This indicates that APP-KPI⁺ is influenced by similar regulatory mechanisms as used by other important anticoagulants. The majority of studies have concentrated on the KPI domain; however the report showing that APP could inhibit the activation of factor XII in a KPI-independent manner (Niwano *et al.*, 1994) suggests that studies on the control of hemostasis by APP should not be restricted to the KPI domain. This inhibitory activity is also increased by the addition of heparin, further emphasizing the importance of these binding sequences in APP function. The involvement of the copper binding site in modulating the anticoagulant activity of APP has not yet been reported. The known effects of copper on APP (see above) and its close proximity to the amino-terminal heparin binding and zinc sites, which are known to influence activity of APP, warrants investigation. Furthermore, a role of copper in coagulation has been established with coagulation factors V and VIII, both of which bind Cu. Cu is also necessary for factor VIII procoagulant activity (Bihoreau *et al.*, 1994, Mann *et al.*, 1984).

Structure of the KPI Domain

While several NMR and other spectroscopic studies have analyzed the A β sequence (Barrow and Zagorski, 1991), the APP-KPI domain is the only reported crystal structure of the APP molecule so far determined. A recombinant fragment encompassing the KPI domain has been crystallized and solved to 1.5 Å resolution (Hynes *et al.*, 1990). The overall fold of the APP-KPI domain is almost identical to the well-characterized KPI containing protein, bovine pancreatic trypsin inhibitor. Three disulfide bonds stabilize its compact pear shaped structure. Two inhibitor binding loops were identified and these correspond to regions of considerable sequence variation among the many (>30) Kunitz inhibitor members. These loops would presumably modulate specificity and affinity. This structural data provides an important molecular framework upon which to understand the anticoagulant activities of the APP-KPI containing isoforms. In an overall context, such studies will add to a better understanding of APP function.

APP AS A MEDIATOR OF NEUROPROTECTION

A considerable body of data has indicated a role for APP in promoting neuronal survival. Exogenously added APP can protect primary neuronal cultures or cell lines from hypoglycemia, glutamate excitotoxicity or A β toxicity (Goodman and Mattson, 1994; Mattson *et al.*, 1993; Schubert and Behl, 1993). Protection appears to occur by APP lowering intracellular calcium ([Ca]_i) levels (Mattson *et al.*, 1993). The current data suggests APP protects cells by activating guanylate cyclase which in turn causes an increase in cGMP levels and an activation of cGMP-dependent protein kinases (Barger *et al.*, 1995). This would lead to stimulation of K⁺ channels which causes the decrease in [Ca]_i levels (Furukawa *et al.*, 1996). An alternate pathway involving activation of the transcription factor NF κ B has also been proposed (Barger and Mattson, 1996).

The region of APP which mediates this effect has been localized by antibody inhibition studies to the carboxy terminus (Mattson *et al.*, 1993). Deletion analysis identifies the active site to amino acids 591–612 (APP695 numbering). This encompasses the amino-terminal portion of the A β sequence and correlates with sAPP α exhibiting up to 100-fold greater neuroprotection than the sAPP β form (Furukawa *et al.*, 1996). The difference between the sAPP α and β forms may indicate a possible function for the different processing events. A role for heparin in neuroprotection has also been raised since heparinase treatment reduces the excitoprotective actions of APP. This may correlate with the heparin binding site contained within the A β sequence (Furukawa *et al.*, 1996). This region of the APP molecule could therefore have important biological functions beyond its involvement in amyloid formation. The finding that the A β sequence is a trafficking signal for axonal sorting in neurons provides strong evidence for this (Tienari *et al.*, 1996).

In vivo evidence for the neuroprotective activity of APP comes from transgenic mice experiments. Mating of APP-transgenic mice with HIV-gp120-transgenic mice resulted in significant protection against HIV-gp 120 induced neurotoxicity (Mucke *et al.*, 1995). Further, intraventricular administration of either APP695 or APP751, following transient ischemia, results in an increased number of surviving CA1 neurons (Smith-Swintosky *et al.*, 1994). This is consistent with other studies showing an increase in APP expression following brain injury (McKenzie *et al.*, 1994, Nakamura *et al.*, 1992). We have investigated the function of the normal endogenous APP in neuroprotection by studying neuronal cultures from APP knockout mice. These studies show there is no difference in neuronal survival between knockout and wild-type cells subjected to different oxidative stresses or excitotoxic insults (Anthony White., Roberto Cappai and Colin L. Masters, manuscript in preparation). This could reflect a redundancy in the APP gene family, since APLP2 expression has been found to be upregulated following neuronal toxicity.

THE FUTURE CHALLENGE

The challenge for those investigating the activities and function of APP is to incorporate all reported activities into a single function of the APP-gene family. Such an effort must include a role for the binding of zinc and copper ions, heparin and other glycoconjugates, together with the expression of the APP-superfamily in many different tissues, both as transmembrane and secreted molecules. A further level of complexity is generated by the diversity of splice variants and post-translational modifications.

Although the large size of the APP protein represents a daunting crystallographic challenge, the rewards of inter-pretng its function in relation to structure are important.

ACKNOWLEDGEMENTS

We acknowledge the support of the Deutsche Forschungsgemeinschaft and the Bundesministerium für Forschung und Technologie and the National Health and Medical Research Council of Australia.

REFERENCES

- Atwood, C.S., Huang, X., Moir, R.D., Tanzi, R.E., and Bush, A.I. (1997) Neurochemical environments that precipitate A β . In K.Iqbal, B.Winblad, T.Nishimura, M.Takeda, and H.M.Wisniewski, (eds.), *Alzheimer's Disease: Biology, Diagnosis and Therapeutics*, John Wiley & Sons Ltd, Chichester, pp. 327–336.
- Barger, S.W., Fiscus, R.R., Ruth, P., Hofmann, F., and Mattson, M.P. (1995) Role of cyclic GMP in the regulation of neuronal calcium and survival by secreted forms of beta-amyloid precursor. *J. Neurochem.*, **64**, 2087–2096.
- Barger, S.W. and Mattson, M.P. (1996) Induction of neuroprotective kappa B-dependent transcription by secreted forms of the Alzheimer's beta-amyloid precursor. *Brain Res. Mol. Brain Res.*, **40**, 116–126.
- Barrow, C., and Zagorski, M. (1991) Solution structures of β peptide and its constituent fragments: relation to amyloid deposition. *Science*, **253**, 179–182.
- Beem, K.M., Rich, W.E., and Rajagopalan, K.V. (1974) Total reconstitution of copper-zinc superoxide dismutase. *J. Biol. Chem.*, **249**, 7298–7305.
- Behr, D., Hesse, L., Masters, C.L., and Multhaup, G. (1996) Regulation of amyloid protein precursor (APP) binding to collagen and mapping of the binding sites on APP and collagen type I. *J. Biol. Chem.*, **271**, 1613–1620.
- Bihoreau, N., Pin, S., de-Kersabiec, A.M., Vidot, E., and Fontaine-Aupart, M.P. (1994) Copper atom identification in the active and inactive forms of plasma-derived FVIII and recombinant FVIII-delta II. *Eur. J. Biochem.*, **222**, 41–48.
- Breen, K.C. (1992) APP-collagen interaction is mediated by a heparin bridge mechanism. *Mol. Chem. Neuropathol.*, **16**, 109–121.
- Brown, R.H., Jr. (1995) Amyotrophic lateral sclerosis: recent insights from genetics and transgenic mice. *Cell*, **80**, 687–692.
- Bush, A.I., Multhaup, G., Moir, R.D., Williamson, T.G., Small, D.H., Rumble, B., Pollwein, P., Beyreuther, K., and Masters, C.L. (1993) A novel zinc(II) binding site modulates the function of the beta A4 amyloid protein precursor of Alzheimer's disease. *J. Biol. Chem.*, **268**, 16109–16112.
- Bush, A.I., Pettingell, W.H., Multhaup, G., d Paradis, M., Vonsattel, J.P., Gusella, J.F., Beyreuther, K., Masters, C.L., and Tanzi, R.E. (1994) Rapid induction of Alzheimer A beta amyloid formation by zinc. *Science*, **265**, 1464–1467.
- Bush, A.I., Pettingell, W.J., de Paradis, M., Tanzi, R.E., and Wasco, W. (1994) The amyloid beta-protein precursor and its mammalian homologues. Evidence for a zinc-modulated heparin-binding superfamily. *J. Biol. Chem.*, **269**, 26618–26621.
- Camakaris, J., Petris, M.J., Bailey, L., Shen, P.Y., Lockhart, P., Glover, T.W., Barcroft, C.L., Patton, J., and Mercer, J.F.B. (1995) Gene amplification of the Menkes (Mnk ATP7a) P Type ATPase gene of CHO cells is associated with copper resistance and enhanced copper efflux. *Hum. Mol. Genet.*, **4**, 2117–2123.

- Clarris, H.J., Cappai, R., Heffernan, D., Beyreuther, K., Masters, C.L., and Small, D.H. (1997) Identification of heparin-binding domains in the amyloid precursor protein of Alzheimer's disease by deletion mutagenesis and peptide mapping. *J. Neurochem.*, **68**, 1164–1172.
- Cole, G.J., and Glaser, L. (1986) A heparin-binding domain from N-CAM is involved in neural cell-substratum adhesion. *J. Cell Biol.*, **102**, 403–12.
- Daigle, I., and Li, C. (1993) apl-1, a *Caenorhabditis elegans* gene encoding a protein related to the human beta-amyloid protein precursor. *Proc. Natl. Acad. Sci. USA*, **90**, 12045–12049.
- Doyle, E., Bruce, M.T., Breen, K.C., Smith, D.C., Anderton, B., and Regan, C.M. (1990) Intra-ventricular infusions of antibodies to amyloid-beta-protein precursor impair the acquisition of a passive avoidance response in the rat. *Neurosci Lett*, **115**, 97–102.
- Dyrks, T., Dyrks, E., Hartmann, T., Masters, C., and Beyreuther, K. (1992) Amyloidogenicity of β A4 and β A4-bearing amyloid protein precursor fragments by metal-catalyzed oxidation. *J. Biol Chem.*, **267**, 18210–18217.
- Fioretti, E., Binotti, I., Barra, D., Citro, G., Ascoli, F., and Anonini, E. (1983) Heterogeneity of the basic pancreatic inhibitor (Kunitz) in various bovine organs. *Eur. J. Biochem.*, **130**, 13–18.
- Franceschi, C., Chiricolo, M., Licastro, F., Zannotti, M., Masi, M., Mocchegiani, E., and Fabris, N. (1988) Oral zinc supplementation in Down's syndrome: restoration of thymic endocrine activity and of some immune defects. *J. Ment. Defic. Res.*, **32**, 169–181.
- Fraser, P.E., Nguyen, J.T., Chin, D.T., and Kirschner, D.A. (1992) Effects of sulfate ions on Alzheimer beta/A4 peptide assemblies: implications for amyloid fibril-proteoglycan interactions. *J. Neurochem.*, **59**, 1531–1540.
- Furukawa, K., Barger, S.W., Blalock, E.M., and Mattson, M.P. (1996) Activation of K⁺ channels and suppression of neuronal activity by secreted beta-amyloid-precursor protein. *Nature*, **379**, 74–78.
- Furukawa, K., Sopher, B.L., Rydel, R.E., Begley, J.G., Pham, D.G., Martin, G.M., Fox, M., and Mattson, M.P. (1996) Increased activity-regulating and neuroprotective efficacy of alpha-secretase-derived secreted amyloid precursor protein conferred by a C-terminal heparin-binding domain. *J. Neurochem.*, **67**, 1882–96.
- Ghisso, J., Rostagno, A., Gardella, J.E., Liem, L., Gorevic, P.D., and Frangione, B. (1992) A 109-amino-acid C-terminal fragment of Alzheimer's-disease amyloid precursor protein contains a sequence, -RHDS-, that promotes cell adhesion. *Biochem. J.*, **288**, 1053–1059.
- 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.
- Goodman, Y., and Mattson, M.P. (1994) Secreted forms of beta-amyloid precursor protein protect hippocampal neurons against amyloid beta-peptide-induced oxidative injury. *Exp. Neurol.*, **128**, 1–12.
- Greenberg, S.M., Selkoe, D.J., Qiu, W.Q., Ben-Itzhak, A., and Kosik, K.S. (1995) Amino-terminal region of the β -amyloid precursor protein activates mitogen-activated protein kinase. *Neurosci. Lett.*, **198**, 52–56.
- 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., Deng, H.X., Chen, W., Zhai, P., Sufit, R.L., and Siddique, T. (1994) Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation. *Science*, **264**, 1772–1775.
- Haass, C., Schlossmacher, M.G., Hung, A.Y., Vigo Pelfrey, C., Mellon, A., Ostaszewski, B.L., Lieberburg, I., Koo, E.H., Schenk, D., Teplow, D.B., and Selkoe, D.J. (1992) Amyloid beta-peptide is produced by cultured cells during normal metabolism. *Nature*, **359**, 322–325.
- Harris, E.D. (1991) Copper transport: an overview. *Proc. Soc. Exp. Biol Med.*, **196**, 130–40.

- Hesse, L., Beher, D., Masters, C.L., and Multhaup, G. (1994) The beta A4 amyloid precursor protein binding to copper. *FEBS. Lett.*, **349**, 109–116.
- Hodgson, E.K., and Fridovich, I. (1975) The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide: chemiluminescence and peroxidation. *Biochemistry*, **14**, 5299–5303.
- Huber, G., Martin, J.R., Loffler, J., and Moreau, J.L. (1993) Involvement of amyloid precursor protein in memory formation in the rat: an indirect antibody approach. *Brain Res*, **603**, 348–352.
- Hung, I.H., Suzuki, M., Yamaguchi, Y., Yuan, D.S., Klausner, R.D., and Gitlin, J.D. (1997) Bio-chemical characterization of the wilson disease protein and functional expression in the yeast *saccharomyces cerevisiae*. *J. Biol. Chem.*, **272**, 21461–2146.
- Hynes, T.R., Randal, M., Kennedy, L.A., Eigenbrot, C., and Kossiakoff, A.A. (1990) X-ray crystal structure of the protease inhibitor domain of Alzheimer's amyloid beta-protein precursor. *Bio-chemistry*, **29**, 10018–10022.
- Ikeo, K., Takahashi, K., and Gojobori, T. (1992) Evolutionary origin of a kunitz-type trypsin inhibitor domain inserted in the amyloid b precursor protein of Alzheimer's disease. *J. Mol. Evol.*, **34**, 536–543.
- Ishida, A., Furukawa, K., Keller, J.N., and Mattson, M.P. (1997) Secreted form of β -amyloid precursor protein shifts the frequency dependency of LTD, and enhances LTP in hippocampal slices. *Neuroreport*, **8**, 2133–2137.
- Kang, J., Lemaire, H.G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grzeschik, K.H., Multhaup, G., Beyreuther, K., and Mueller-Hill, B. (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature*, **325**, 733–736.
- Klier, F.G., Cole, G., Stallcup, W., and Schubert, D. (1990) Amyloid beta-protein precursor is associated with extracellular matrix. *Brain Res.*, **515**, 336–342.
- Knauer, D.J., and Cunningham, D.D. (1982) Epidermal growth factor carrier protein binds to cells via a complex with released carried protein nexin. *Proc. Natl. Acad. Sci. USA*, **79**, 2310–2314.
- Knauer, D.J., Thompson, J.A., and Cunningham, D.D. (1983) Protease nexins: cell-secreted proteins that mediate the binding, internalization, and degradation of regulatory serine proteases. *J. Cell. Physiol.*, **117**, 385–396.
- Knauer, M.F., Orlando, R.A., and Glabe, C.G. (1996) Cell surface APP751 forms complexes with protease nexin 2 ligands and is internalized via the low density lipoprotein receptor-related protein (LRP). *Brain Res.*, **740**, 6–14.
- Komiyama, Y., Murakami, T., Egawa, H., Okubo, S., Yasunaga, K., and Murata, K. (1992) Purification of factor XIa inhibitor from human platelets. *Thromb. Res.*, **66**, 397–408.
- Koo, E.H., and Squazzo, S.L. (1994) Evidence that production and release of amyloid beta-protein involves the endocytic pathway. *J. Biol. Chem.*, **269**, 17386–17389.
- Kounnas, M.Z., Moir, R.D., Rebeck, G.W., Bush, A.I., Argraves, W.S., Tanzi, R.E., Hyman, B.T., and Strickland, D.K. (1995) LDL receptor-related protein, a multifunctional ApoE receptor, binds secreted beta-amyloid precursor protein and mediates its degradation. *Cell*, **82**, 331–340.
- Kozlowski, M.R., Spanoyannis, A., Manly, S.P., Fidel, S.A., and Neve, R.L. (1992) The neurotoxic carboxy-terminal fragment of the Alzheimer amyloid precursor binds specifically to a neuronal cell surface molecule: pH dependence of the neurotoxicity and the binding. *J. Neurosci.*, **12**, 1679–1687.
- Li, Q.X., Berndt, M.C., Bush, A.I., Rumble, B., Mackenzie, I., Friedhuber, A., Beyreuther, K., and Masters, C.L. (1994) Membrane-associated forms of the beta A4 amyloid protein precursor of Alzheimer's disease in human platelet and brain: surface expression on the activated human platelet. *Blood*, **84**, 133–142.
- Li, Q.X., Evin, G., Small, D.H., Multhaup, G., Beyreuther, K., and Masters, C.L. (1995) Proteolytic processing of Alzheimer's disease beta A4 amyloid precursor protein in human platelets. *J. Biol. Chem.*, **270**, 14140–14147.

- Lindahl, U., and Höök, M. (1978) Glycosaminoglycans and their binding to biological macromolecules. *Annu. Rev. Biochem.*, **47**, 385–417.
- Linder, M.C., and Hazegh Azam, M. (1996) Copper biochemistry and molecular biology. *Am. J. Clin. Nutr.*, **63**, 797s–811s.
- Mahdi, F., Van Nostrand, W.E., and Schmaier, A.H. (1995) Protease nexin-2/amyloid beta-protein precursor inhibits factor Xa in the prothrombinase complex. *J. Biol. Chem.*, **270**, 23468–23474.
- Mann, K.G., Lawler, C.M., Vehar, G.A., and Church, W.R. (1984) Coagulation factor V contains copper ions. *J. Biol. Chem.*, **259**, 12949–12951.
- Mantyh, P.W., Ghilardi, J.R., Rogers, S., DeMaster, E., Allen, C.J., Stimson, E.R., and Maggio, J.E. (1993) Aluminum, iron, and zinc ions promote aggregation of physiological concentrations of beta-amyloid peptide. *J. Neurochem.*, **61**, 1171–1174.
- Margalit, H., Fischer, N., and Ben Sasson, S.A. (1993) Comparative analysis of structurally defined heparin binding sequences reveals a distinct spatial distribution of basic residues. *J. Biol. Chem.*, **268**, 19228–19231.
- Mattson, M.P., Cheng, B., Culwell, A.R., Esch, F.S., Lieberburg, I., and Rydel, R.E. (1993) Evidence for excitoprotective and intraneuronal calcium-regulating roles for secreted forms of the beta-amyloid precursor protein. *Neuron*, **10**, 243–254.
- McKenzie, J.E., Gentleman, S.M., Roberts, G.W., Graham, D.I., and Royston, M.C. (1994) Increased numbers of beta APP-immunoreactive neurones in the entorhinal cortex after head injury. *Neuroreport.*, **6**, 161–164.
- Milward, E.A., Papadopoulos, R., Fuller, S.J., Moir, R.D., Small, D., Beyreuther, K., and Masters, C.L. (1992) The amyloid protein precursor of Alzheimer's disease is a mediator of the effects of nerve growth factor on neurite outgrowth. *Neuron*, **9**, 129–137.
- Miyata, M. and Smith, J.D. (1996) Apolipoprotein E allele-specific antioxidant activity and effects on cytotoxicity by oxidative insults and beta-amyloid peptides. *Nat. Genet.*, **14**, 55–61.
- Mucke, L., Abraham, C.R., Ruppe, M.D., Rockenstein, E.M., Toggas, S.M., Mallory, M., Alford, M., and Masliah, E. (1995) Protection against HIV-1 gp120-induced brain damage by neuronal expression of human amyloid precursor protein. *J. Exp. Med.*, **181**, 1551–1556.
- Mucke, L., Masliah, E., Johnson, W.B., Ruppe, M.D., Alford, M., Rockenstein, E.M., S., F.-P., Pietropaolo, M., Mallory, M., and Abraham, C.R. (1994) Synaptotrophic effects of human amyloid beta protein precursors in the cortex of transgenic mice. *Brain Res.*, **666**, 151–167.
- Multhaup, G. (1994) Identification and regulation of the high affinity binding site of the Alzheimer's disease amyloid protein precursor (APP) to glycosaminoglycans. *Biochimie*, **76**, 304–311.
- Multhaup, G., Bush, A.I., Pollwein, P., and Masters, C.L. (1994) Interaction between the zinc(II) and the heparin binding site of the Alzheimer's disease beta A4 amyloid precursor protein (APP). *FEBS Lett.*, **355**, 151–154.
- Multhaup, G., Schlicksupp, A., Hesse, L., Behr, D., Ruppert, T., Masters, C.L., and Beyreuther, K. (1996) The amyloid precursor protein of Alzheimer's disease in the reduction of copper(II) to copper(I). *Science*, **271**, 1406–1409.
- Multhaup, G., Ruppert, T., Schlicksupp, A., Hesse, L., Bill, E., Pipkorn, R., Masters, C.L. and Beyreuther, K. (1998) Copper-binding amyloid precursor protein undergoes a site-specific fragmentation in the reduction of hydrogen peroxide. *Biochemistry*, in press.
- Nakamura, Y., Takeda, M., Niigawa, H., Hariguchi, S., and Nishimura, T. (1992) Amyloid betaprotein precursor deposition in rat hippocampus lesioned by ibotenic acid injection. *Neurosci. Lett.*, **136**, 95–98.
- Napolitano, G., Palka, G., Grimaldi, S., Giuliani, C., Laglia, G., Calabrese, G., Satta, M.A., Neri, G., and Monaco, F. (1990) Growth delay in Down syndrome and zinc sulphate supplementation. *Am. J. Med. Genet. Suppl.*, **7**, 63–65.

- Narindrasorasak, S., Lowery, D.E., Altman, R.A., Gonzalez DeWhitt, P.A., Greenberg, B.D., and Kisilevsky, R. (1992) Characterization of high affinity binding between laminin and Alzheimer's disease amyloid precursor proteins. *Lab. Invest.*, **67**, 643–652.
- Ninomiya, H., Roch, J.M., Sundsmo, M., Otero, D.A.C., and Saitoh, T. (1993) Amino acid sequence RERMS represents the active domain of amyloid b/A4 protein precursor that promotes fibroblast growth. *J. Cell. Biol.*, **121**, 879–886.
- Niwan, H., Embury, P.B., Greenberg, B.D., and Ratnoff, O.D. (1994) Inhibitory action of amyloid precursor protein against human Hageman factor (factor XII). *J. Lab. Clin. Med.*, **125**, 251–256.
- Okamoto, T., Takeda, S., Giambarella, U., Murayama, Y., Matsui, T., Katada, T., Matsuura, Y., and Nishimoto, I. (1996) Intrinsic signalling function of APP as a novel target of three V642 mutations linked to familial Alzheimer's disease. *EMBO J.*, **15**, 3769–3777.
- Oltersdorf, T., Fritz, L.C., Schenk, D.B., Lieberburg, I., Johnson-Wood, K.I., Beattie, E.C., Ward, P.J., Blacher, R.W., Dovey, H.F., and Sinha, S. (1989) The secreted form of the Alzheimer's amyloid precursor protein with the Kunitz domain is protease nexin-II. *Nature*, **341**, 144–147.
- Ooi, C.E., Rabinovich, E., Dancis, A., Bonifacio, J.S., and Klausner, R.D. (1996) Copper-dependent degradation of the *Saccharomyces cerevisiae* plasma membrane copper transporter Ctr1p in the apparent absence of endocytosis. *EMBO J.*, **15**, 3515–3523.
- Percival, S.S. and Harris, E.D. (1989) Ascorbate enhances copper transport from ceruloplasmin into human K562 cells. *J. Nutr.*, **119**, 779–784.
- Roch, J.M., Shapiro, I.P., Sundsmo, M.P., Otero, D.A., Refolo, L.M., Robakis, N.K., and Saitoh, T. (1992) Bacterial expression, purification, and functional mapping of the amyloid beta/A4 protein precursor. *J. Biol. Chem.*, **267**, 2214–2221.
- Rosen, D.R., Martin-Morris, L., Luo, L.Q., and White, K. (1989) A *Drosophila* gene encoding a protein resembling the human beta-amyloid protein precursor. *Proc. Natl. Acad. Sci. USA*, **86**, 2478–2482.
- Rosenberg, R.D. (1985) Role of heparin and heparinlike molecules in thrombosis and atherosclerosis. *Fed-Proc.*, **44**, 404–409.
- Saitoh, T., Sundsmo, M., Roch, J.M., Kimura, N., Cole, G., Schubert, D., Oltersdorf, T., and Schenk, D.B. (1989) Secreted form of amyloid beta protein precursor is involved in the growth regulation of fibroblasts. *Cell*, **58**, 615–622.
- Sandbrink, R., Masters, C.L., and Beyreuther, K. (1994) Complete nucleotide and deduced amino acid sequence of rat amyloid protein precursor-like protein 2 (APLP2/APPH): two amino acids length difference to human and murine homologues. *Biochim. Biophys. Acta.*, **1219**, 167–170.
- Saporito-Irwin, S.M. and Van Nostrand, W.E. (1995) Coagulation factor XIa cleaves the RHDS sequence and abolishes the cell adhesive properties of the amyloid β -protein. *J. Biol. Chem.*, **270**, 26265–26269.
- Sato, K., Akaike, T., Kohno, M., Ando, M., and Maeda, H. (1992) Hydroxyl radical production by H₂O₂ plus Cu,Zn-superoxide dismutase reflects the activity of free copper released from the oxidatively damaged enzyme. *J. Biol. Chem.*, **267**, 25371–25377.
- Scandura, J.M., Zhang, Y., Van Nostrand, W.E., and Walsh, P.N. (1997) Progress curve analysis of the kinetics with which blood coagulation factor XIa is inhibited by protease nexin-2. *Biochemistry*, **36**, 412–420.
- Schmaier, A.H., Dahl, L.D., Rozemuller, A.J., Roos, R.A., Wagner, S.L., Chung, R., and Van Nostrand, W.E. (1993) Protease nexin-2/amyloid beta protein precursor. A tight-binding inhibitor of coagulation factor IXa. *J. Clin. Invest.*, **92**, 2540–5.

- Schubert, D. and Behl, C. (1993) The expression of amyloid beta protein precursor protects nerve cells from beta-amyloid and glutamate toxicity and alters their interaction with the extracellular matrix. *Brain Res.*, **629**, 275–282.
- Schubert, D., Cole, G., Saitoh, T., and Oltersdorf, T. (1989) Amyloid beta precursor is a mitogen. *Biochem. Biophys. Res. Commun.*, **162**, 52–56.
- Schubert, D., Jin, L.-W., Saitoh, T., and Cole, G. (1989) The regulation of amyloid β protein precursor secretion and its modulatory role in cell adhesion. *Neuron*, **3**, 689–694.
- Schubert, D., LaCorbiere, M., Saitoh, T., and Cole, G. (1989) Characterization of an amyloid beta precursor protein that binds heparin and contains tyrosine sulfate. *Proc. Natl. Acad. Sci. USA*, **86**, 2066–9.
- Shioi, J., Pangalos, M.N., Ripellino, J.A., Vassilacopoulou, D., Mytilineou, C., Margolis, R.U., and Robakis, N.K. (1995) The Alzheimer amyloid precursor proteoglycan (appican) is present in brain and is produced by astrocytes but not by neurons in primary neural cultures. *J. Biol. Chem.*, **270**, 11839–11844.
- Shivers, B.D., Hilbich, C., Multhaup, G., Salbaum, M., Beyreuther, K., and Seeburg, P.H. (1988) Alzheimer's disease amyloidogenic glycoprotein: expression pattern in rat brain suggests a role in cell contact. *EMBO. J.*, **7**, 1365–1370.
- Simons, M., Ikonen, E., Tienari, P.J., Cidarregui, A., Monning, U., Beyreuther, K., and Dotti, C.G. (1995) Intracellular routing of human amyloid protein precursor: Axonal delivery followed by transport to the dendrites. *J. Neurosci. Res.*, **41**, 121–128.
- Sisodia, S.S., Koo, E.H., Beyreuther, K., Unterbeck, A., and Price, D.L. (1990) Evidence that betaamyloid protein in Alzheimer's disease is not derived by normal processing. *Science*, **248**, 492–495.
- Slunt, H.H., Thinakaran, G., Von, K.C., Lo, A.C., Tanzi, R.E., and Sisodia, S.S. (1994) Expression of a ubiquitous, cross-reactive homologue of the mouse beta-amyloid precursor protein (APP). *J. Biol. Chem.*, **269**, 2637–2644.
- Small, D.H., Nurcombe, V., Moir, R., Michaelson, S., Monard, D., Beyreuther, K., and Masters, C.L. (1992) Association and release of the amyloid protein precursor of Alzheimer's disease from chick brain extracellular matrix. *J. Neurosci.*, **12**, 4143–4150.
- Small, D.H., Nurcombe, V., Reed, G., Clariss, H., Moir, R., Beyreuther, K., and Masters, C.L. (1994) A heparin-binding domain in the amyloid protein precursor of Alzheimer's disease is involved in the regulation of neurite outgrowth. *J. Neurosci.*, **14**, 2117–2127.
- Smith, R.P., Higuchi, D.A., and Broze, G.J. (1990) Platelet coagulation factor XIa-inhibitor, a form of Alzheimer amyloid precursor protein. *Science*, **248**, 1126–1128.
- Smith-Swintosky, V.L., Pettigrew, L.C., Craddock, S.D., Culwell, A.R., Rydel, R.E., and Mattson, M.P. (1994) Secreted forms of beta-amyloid precursor protein protect against ischemic brain injury. *J. Neurochem.*, **63**, 781–784.
- Snow, A.D., Kinsella, M.G., Parks, E., Sekiguchi, R.T., Miller, J.D., Kimata, K., and Wight, T.N. (1995) Differential binding of vascular cell-derived proteoglycans (perlecan, biglycan, decorin, and versican) to the beta-amyloid protein of Alzheimer's disease. *Arch. Biochem. Biophys.*, **320**, 84–95.
- Sprecher, C.A., Grant, F.J., Grimm, G., O'Hara, P.J., Norris, F., Norris, K., and Foster, D.C. (1993) Molecular cloning of the cDNA for a human amyloid precursor protein homolog: evidence for a multigene family. *Biochemistry*, **32**, 4481–4486.
- Stewart, L.C. and Klinman, J.P. (1988) Dopamine beta-hydroxylase of adrenal chromaffin granules: structure and function. *Annu. Rev. Biochem.*, **57**, 551–592.

- Tienari, P.J., De, S.B., Ikonen, E., Simons, M., Weidemann, A., Czech, C., Hartmann, T., Ida, N., Multhaup, G., Masters, C.L., Van, L.F., Beyreuther, K., and Dotti, C.G. (1996) The beta-amyloid domain is essential for axonal sorting of amyloid precursor protein. *EMBO J*, **15**, 5218–5229.
- Van Nostrand, W.E. (1995) Zinc (II) selectively enhances the inhibition of coagulation factor XIa by protease nexin-2/amyloid beta-protein precursor. *Thromb. Res.*, **78**, 43–53.
- Van Nostrand, W.E. and Cunningham, D.D. (1987) Purification of protease nexin II from human fibroblasts. *J. Biol. Chem.*, **262**, 8508–8514.
- Van Nostrand, W.E., Schmaier, A.H., Farrow, J.S., and Cunningham, D.D. (1990) Protease nexinII (amyloid beta-protein precursor): a platelet alpha-granule protein. *Science*, **248**, 745–748.
- Van Nostrand, W.E., Schmaier, A.H., Neiditch, B.R., Siegel, R.S., Raschke, W.C., Sisodia, S.S., and Wagner, S.L. (1994) Expression, purification, and characterization of the Kunitz-type proteinase inhibitor domain of the amyloid beta-protein precursor-like protein-2. *Biochim. Biophys. Acta.*, **1209**, 165–170.
- Van Nostrand, W.E., Schmaier, A.H., Siegel, R.S., Wagner, S.L., and Raschke, W.C. (1995) Enhanced plasmin inhibition by a reactive center lysine mutant of the Kunitz-type protease inhibitor domain of the amyloid beta-protein precursor. *J. Biol. Chem*, **270**, 22827–22830.
- Van Nostrand, W.E., Wagner, S.L., Farrow, J.S., and Cunningham, D.D. (1990) Immunopurification and protease inhibitory properties of protease nexin-2/amyloid beta-protein precursor. *J. Biol. Chem.*, **265**, 9591–9594.
- Van Nostrand, W.E., Wagner, S.L., Suzuki, M., Choi, B.H., Farrow, J.S., Geddes, J.W., Cotman, C.W., and Cunningham, D.D. (1989) Protease nexin-II, a potent antichymotrypsin, shows identity to amyloid beta-protein precursor. *Nature*, **341**, 546–549.
- Vidal, F., Blangy, A., Rassoulzadegan, M., and Cuzin, F. (1992) A murine sequence-specific DNA binding protein shows extensive local similarities to the amyloid precursor protein. *Biochem. Biophys. Res. Commun.*, **189**, 1336–1341.
- Wagner, S.L., Siegel, R.S., Vedvick, T.S., Raschke, W.C., and Van Nostrand, W.E. (1992) High level expression, purification, and characterization of the Kunitz-type protease inhibitor domain of protease nexin-2/amyloid beta-protein precursor. *Biochem. Biophys. Res. Commun.*, **186**, 1138–1145.
- Wasco, W., Bupp, K., Magendantz, M., Gusella, J.F., Tanzi, R.E., and Solomon, F. (1992) Identification of a mouse brain cDNA that encodes a protein related to the Alzheimer disease-associated amyloid beta protein precursor. *Proc. Natl. Acad. Sci. USA*, **89**, 10758–10762.
- Whyte, S., Jones, L., Coulson, E.J., Moir, R.D., Bush, A.I., Beyreuther, K., and Masters, C.L. (1997) The metabolism of the amyloid precursor protein of Alzheimer's disease and dietary zinc. In K. Iqbal, B. Winblad, T. Nishimura, M. Takeda, and H.M. Wisniewski, (eds.), *Alzheimer's Disease: Biology, Diagnosis and Therapeutics*, John Wiley & Sons Ltd, Chichester, pp. 417–422.
- Wiedau-Pazos, M., Goto, J.J., Rabizadeh, S., Gralla, E.B., Roe, J.A., Lee, M.K., Valentine, J.S., and Bredesen, D.E. (1996) Altered reactivity of superoxide dismutase in familial amyotrophic lateral sclerosis. *Science*, **271**, 515–518.
- Williamson, T.G., Mok, S.S., Henry, A., Cappai, R., Lander, A.D., Nurcombe, V., Beyreuther, K., Masters, C.L., and Small, D.H. (1996) Secreted glypican binds to the amyloid precursor protein of Alzheimer's disease (APP) and inhibits APP-induced neurite outgrowth. *J. Biol. Chem.*, **271**, 31215–31221.
- 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.

- Wu, A., Pangalos, M.N., Efthimiopoulos, S., Shioi, J., and Robakis, N.K. (1997) Appican expression induces morphological changes in C6 glioma cells and promotes adhesion of neural cells to the extracellular matrix. *J. Neurosci.*, **17**, 4987–4993.
- Yamada, T., Sasaki, H., Furuya, H., Miyata, T., Goto, I., and Sakaki, Y. (1987) Complementary DNA for the mouse homolog of the human amyloid beta protein precursor. *Biochem. Biophys. Res. Commun.*, **149**, 665–671.
- Yamazaki, T., Selkoe, D.J., and Koo, E.H. (1995) Trafficking of cell surface beta-amyloid precursor protein: retrograde and transcytotic transport in cultured neurons. *J. Cell. Biol.*, **129**, 431–442.
- Yim, H.S., Kang, J.H., Chock, P.B., Stadtman, E.R., and Yim, M.B. (1997) A familial amyotrophic lateral sclerosis-associated A4V Cu,Zn-Superoxide dismutase mutant has a lower K-m for hydrogen peroxide. *J. Biol. Chem.*, **272**, 8861–8863.
- Yim, M.B., Kang, J.H., Yim, H.S., Kwak, H.S., Chock, P.B., and Stadtman, E.R. (1996) A gain-of-function of an amyotrophic lateral sclerosis-associated Cu,Zn-superoxide dismutase mutant: An enhancement of free radical formation due to a decrease in K-m for hydrogen peroxide. *Proc. Natl. Acad. Sci. USA*, **93**, 5709–5714.
- Yurchenco, P.D., Cheng, Y.S., and Schittny, J.C. (1990) Heparin modulation of laminin polymerization. *J. Biol. Chem.*, **265**, 3981–3991.

5. PROTEOLYTIC PROCESSING OF THE β -AMYLOID PRECURSOR PROTEIN

MARTIN CITRON^{1,2} and DENNIS J. SELKOE¹

¹*Center for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical School, Boston, MA*

²*AMGEN Inc., Dept. of Neuroscience, Thousand Oaks, CA*

INTRODUCTION

Few macromolecules have become the object of more intense chemical and biological scrutiny in recent years than the β -amyloid precursor protein (APP). The reason for this attention is clear. Proteolytic processing of APP leads to the production of the major component of Alzheimer's disease (AD) amyloid, the 39–43 amino acid amyloid β -peptide (A β). Accumulating evidence points to an early and—at least for early onset familial AD—causative role of A β in the pathogenesis of the disease (see the chapter by Younkin). This realization has stimulated a lot of basic research on APP processing. At the same time, therapeutic strategies aimed at lowering A β production have gained increasing interest, and the first A β lowering agents are likely to enter clinical trials within the next year or two. Here, we review the complex topic of APP processing, with particular emphasis on the proteases involved and on results published within the last two years.

The Amyloid β -Precursor Protein

In 1984, Glenner and Wong first isolated and purified the subunit protein of the meningo-vascular amyloid filaments in AD and determined its amino-terminal sequence (Glenner and Wong, 1984). They named this novel protein the amyloid β -peptide (A β). Shortly thereafter, compacted amyloid plaque cores were partially purified from AD cerebral cortex and shown to consist of essentially the same A β peptide (Masters *et al.*, 1985; Gorevic *et al.*, 1986; Roher *et al.*, 1986; Selkoe *et al.*, 1986). Based on the partial amino acid sequence, cDNAs encoding part or all of the A β precursor were cloned (Goldgaber *et al.*, 1987; Kang *et al.*, 1987; Robakis *et al.*, 1987; Tanzi *et al.*, 1987). The deduced amino acid sequence obtained from a full-length cDNA predicted a 695 amino acid type I transmembrane protein containing a 17 residue signal peptide, a single membrane-spanning region and a short cytoplasmic tail. The A β sequence was predicted to begin 28 residues amino-terminal to the transmembrane domain and extend 11–15 residues into that domain ([Figure 1](#)). Various major isoforms of APP result from alternative splicing of at least three exons in the APP gene. The most

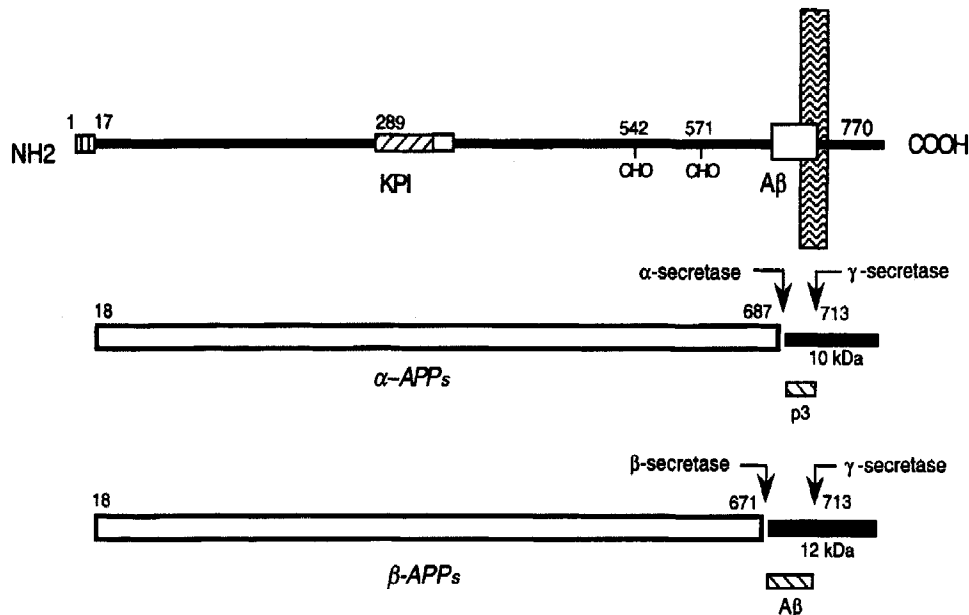


Figure 1. Schematic diagrams of the β -amyloid precursor protein and its principal metabolic derivatives. The upper diagram depicts the largest of the known APP alternate transcripts, comprising 770 amino acids. Regions of interest are indicated at their correct relative positions. A 17-residue signal peptide occurs at the amino terminus (box with vertical lines). Two alternatively spliced exons of 56 and 19 amino acids are inserted at residue 289; the first contains a serine protease inhibitor domain of the Kunitz type (KPI). Two sites of N-glycosylation (CHO) are found at residues 542 and 571. A single membrane-spanning domain at amino acids 700–723 is indicated by the vertical hatched bar. The amyloid β -protein (A β) fragment (white box) includes 28 residues just outside the membrane plus the first 12–14 residues of the transmembrane domain. In the middle diagram, the arrow indicates the site (after residue 687) of a constitutive proteolytic cleavage made by an unknown protease(s) designated α -secretase that enables secretion of the large, soluble ectodomain of APP (α -APP_s) into the medium and retention of the 83 residue carboxy-terminal fragment (~10 kDa) in the membrane. The 10 kDa fragment can undergo cleavage by an unknown protease(s) called γ -secretase at residue 711 or residue 713 to release the p3 peptides. The lower diagram depicts the alternative proteolytic cleavage after residue 671 by an unknown enzyme(s) called β -secretase that results in the secretion of a truncated APP_s (β -APP_s) molecule and the retention of a 99 residue (~12 kDa) carboxy-terminal fragment. The 12 kDa fragment can also undergo cleavage by γ -secretase to release the A β peptides.

widely and abundantly expressed of these is the 751 amino acid form containing exon 7, which encodes a 56 amino acid region with structural and functional properties of a serine protease inhibitor of the Kunitz-type (KPI) (Kitaguchi *et al.*, 1988; Ponte *et al.*, 1988; Tanzi *et al.*, 1988). The isoform of APP which is selectively expressed in neurons lacks exon 7 and contains 695 amino acids. In contrast, astrocytes and microglia selectively express isoforms lacking exon 15, which encodes a small region prior to the transmembrane domain (called L-APP isoforms) (Konig *et al.*, 1992).

A variety of posttranslational modifications of APP have been described in cultured cells. After synthesis, APP is first N-glycosylated in the endoplasmic reticulum (ER) and then N- and O-glycosylated in the Golgi. Surprisingly 70–80% of the immature N-glycosylated APP are degraded, probably within the ER, and only 20–30% reach the Golgi (Kuentzel *et al.*, 1993; Citron *et al.*, 1996a).

During transit from the endoplasmic reticulum to the cell surface, APP also undergoes sulfation (Weidemann *et al.*, 1989; Oltersdorf *et al.*, 1990) and phosphorylation (Oltersdorf *et al.*, 1990). This constitutive phosphorylation appears to be restricted to the ectodomain (Hung and Selkoe, 1994) and occurs within a post-Golgi compartment and at the cell surface (Walter *et al.*, 1996).

Endoproteolysis of APP

β -secretase cleavage

Early studies showed that cultured neurons as well as various transfected cell-lines that overexpressed APP released the large, soluble ectodomain (designated APP_S) into the medium (Schubert *et al.*, 1989; Weidemann *et al.*, 1989; Esch *et al.*, 1990; Sisodia *et al.*, 1990). This cleavage leaves a ca. 10 kDa carboxyl-terminal fragment (CTF) within the cell (Selkoe *et al.*, 1988) (Figure 1). The percentage of newly synthesized APP that undergoes this secretory cleavage varies widely among cell types but does not seem to exceed about a third (Weidemann *et al.*, 1989). APP_S was also identified in human CSF and brain tissue (Weidemann *et al.*, 1989; Palmert *et al.*, 1989) in both AD and normal control subjects. The finding that protease nexin II, a soluble protease inhibitor involved in cell growth regulation, was identical to an APP_S isoform containing the KPI domain provided additional evidence for a normal secretion event (Oltersdorf *et al.*, 1989; van Nostrand *et al.*, 1989).

Using cell lines transfected with various APP constructs containing all or parts of A β , it was demonstrated that the cleavage releasing conventional APP_S occurred within the exoplasmic portion of A β (Sisodia *et al.*, 1990). The precise cleavage site was then determined by direct protein sequencing of the 10 kDa CTF (Esch *et al.*, 1990). In cells transfected with APP, the soluble form ended at Gln 15 of A β and the amino-terminus of the CTF was Leu 17, suggesting that Lys 16 was cleaved from either fragment by an ectopeptidase. The putative endoprotease involved in APP secretion was named a-secretase. It seems clear that a-secretase cleaves the Gln15-Lys16 bond, Lys16-Leu17 bond or both in various cell lines (Esch *et al.*, 1990; Wang *et al.*, 1991; Anderson *et al.*, 1991; Lowery *et al.*, 1991), except in Down's syndrome fibroblasts where CTFs were found to begin at Phe19, Glu22 and Gly25 (Zhong *et al.*, 1994).

While nobody has definitely identified a-secretase, it remains the best understood APP processing enzyme, a-secretase cleavage occurs both intracellularly (De Strooper *et al.*, 1992; De Strooper *et al.*, 1993) and at the cell surface (Sisodia, 1992). Various studies have addressed the sequence specificity of the secretase. It was reported that amino acid substitutions around the cleavage site and a large deletion mutation that removed the α -secretase cleavage site entirely, did not prevent APP_S generation. In the deletion mutant, cleavage was shifted to a Glu-Val bond 12 amino acids N-terminal to the transmembrane domain, the same distance found in the normal Lys16-Leu17 cleavage. This finding suggested that α -secretase was more distance- than sequence-dependent (Maruyama *et al.*, 1991). When the residues around the Lys16-Leu17 cleavage site were systematically mutated, substitution of Val at the P1 position, Gly at the P2' and Pro at the P3' position were found to significantly reduce α -secretase cleavage. Insertion of 3 amino acids C-terminal to the α -secretase site concomitantly increased the size of the released APP_S molecule (Sisodia, 1992). These findings are consistent with α -

secretase cleaving at a particular distance from the membrane, as well as having some amino acid specificity. In contrast, another study in a different cell system has shown 60% of cleavage at Lys16–Leu17, but 40% of cleavage at Phe19–Phe20 (Zhong *et al.*, 1994). This study also featured a series of substitution and deletion constructs around the favored Lys16–Leu17 site, none of which was able to prevent the release of APP_S. Using two deletion mutants C-terminal to the cleavage site, α -secretase cleavage was found to depend on amino acid sequence rather than membrane distance (Zhong *et al.*, 1994).

As in the case of the other secretases (see below), an intrinsic problem in the interpretation of the cell biological assays is the difficulty in distinguishing between a single protease with relaxed specificity and a family of proteases with specific, different activities. A final conclusion about the sequence specificity of α -secretase(s) can only be made once the purified enzyme is available. While this is not the case yet, a cell-free assay has been described (Boseman Roberts *et al.*, 1994). In this assay, a fusion construct containing the last 105 amino acids of APP was expressed in transfected cells. Incubation of membrane preparations from these cells was shown to cause release of the N-terminal portion of the fusion protein in a time and temperature dependent fashion. The activity in these membranes appeared to be an integral membrane metalloprotease:

The regulation of α -secretase has received considerable attention, particularly the effects of agents which activate protein kinase C (PKC). It was demonstrated that APP_S release is increased when cells are directly treated with phorbol ester (Caporaso *et al.*, 1992) or when cells transfected with muscarinic acetylcholine receptors were treated with carbachol (Nitsch *et al.*, 1992). Major progress in our understanding of α -secretion has come from two recent studies employing a mutant CHO-line defective in phorbol ester-induced shedding of the membrane-polypeptides transforming growth factor a precursor (Arribas and Massague, 1995; Arribas *et al.*, 1996). Interestingly, this mutant line is also defective in the secretion of L-selectin, IL-6 receptor α -subunit, APP and a whole set of other CHO cell surface proteins. The phorbol ester effect could also be blocked by treatment with metalloprotease inhibitors. Since independent mutagenesis and selection experiments yielded mutants having the same recessive phenotype and belonging to the same complementation group, it appears that α -secretase belongs to a common system for membrane protein ectodomain shedding, whose ability to act can be disrupted by recessive mutations in a single gene.

The generation of A β

Because α -secretion involves a cleavage within the A β region, it was concluded that AP must be generated in a different and presumably pathological pathway. Based on this assumption several studies focused on finding CTFs which could serve as amyloidogenic precursors for A β generation. Golde *et al.* (1992) demonstrated that APP transfected cells treated with certain inhibitors of lysosomal proteolysis accumulated CTFs containing the intact A β region. Fragments with these properties were also detected in postmortem human brain (Estus *et al.*, 1992; Nordstedt *et al.*, 1991; Tamaoka *et al.*, 1992). Full-length APP that had been reinternalized from the cell surface plus a whole array of A β containing CTFs could be detected in late endosome/lysosome fractions (Haass *et al.*, 1992a). The reinternalization pathway for APP was recently directly visualized by following the localization and distribution of APP monoclonal antibodies added to intact APP-transfected CHO cells using

immunofluorescence and immunoelectron microscopy. This study demonstrated the rapid recycling of endocytosed APP to the cell surface and its ultimate targeting to lysosomes (Yamazaki *et al.*, 1996). Collectively, these studies proved the existence of pathways that generate APP fragments containing the intact A β region. This still left the problem of cleavage at the membrane-embedded C-terminus of A β . It was generally assumed that this C-terminal cleavage could only follow upon membrane injury. Therefore, it was quite surprising when the secretion of soluble A β into the conditioned media of apparently healthy cells was first reported. These studies indicated that a minor proportion of all APP molecules are turned over to produce secreted A β (Busciglio *et al.*, 1993; Haass *et al.*, 1992b; Seubert *et al.*, 1992; Shoji *et al.*, 1992). A β was also detected in normal cerebrospinal fluid and plasma of humans and other mammals (Seubert *et al.*, 1992). The finding that A β is constitutively produced and secreted has sparked a number of studies addressing various aspects of A β generation, including mechanistic studies and screens for inhibitors of A β generation. In addition to A β , a smaller peptide containing a portion of A β , designated p3, is secreted. This peptide was found to start at position 17 of A β , immediately suggesting that it may be derived from the 10 kDa CTF which remains in the cell after α -secretase cleavage of APP (Haass *et al.*, 1992b). The enzyme(s) involved in generating the amino terminus of A β has been designated β -secretase(s); the enzyme(s) that generate the C-terminus of A β and p3 has been designated γ -secretase(s) (see Figure 1). By analyzing the effects of pharmacological treatments, mutations, etc. on A β and p3, it has been possible to distinguish effects on α -, β -, and γ -secretase cleavage events, assuming that the C-termini of A β and p3 are generated by the same γ -secretase. However, despite intense efforts, there have been no studies that identify β - and γ -secretases at the biochemical level.

β -secretase cleavage

Shortly after the discovery of secreted A β , it was shown that agents which alter intracellular pH, such as chloroquine (Shoji *et al.*, 1992) or ammonium chloride (Haass *et al.*, 1993), lead to a substantial decrease in A β but not p3 secretion, suggesting the importance of an acidic intracellular compartment for β -secretase cleavage. It seems unlikely that β -secretase cleavage happens within lysosomes per se, because leupeptin fails to prevent A β production, and I cells, although defective in lysosomal function, still produce A β (Haass *et al.*, 1993). In contrast, agents like monensin and brefeldin A, which interfere with Golgi function, inhibit the secretion of A β (Haass *et al.*, 1993). It now seems clear that a major route of β -secretase cleavage of the wild-type precursor protein involves the endocytic pathway, because deletion of the cytoplasmic tail of APP, which contains a consensus motif for coated pit-mediated internalization (Chen *et al.*, 1990), decreases the amount of secreted A β , but not p3, indicating that the effect is not at the level of γ -secretase cleavage (Haass *et al.*, 1993; Koo and Squazzo, 1994). Furthermore, potassium depletion, which leads to decreased APP reinternalization, lowers A β secretion. Most importantly, radiolabeled A β can be recovered in the medium following selective cell surface radioiodination of APP (Koo and Squazzo, 1994). However, β -secretase cleavage also occurs in the secretory pathway. This can be inferred from the fact that the cytoplasmic deletion decreases, but does not abolish β -secretase cleavage (Haass *et al.*, 1993). Moreover, Seubert *et al.* (1992) demonstrated the existence of APP_s- β , the large secreted ectodomain of APP cleaved precisely at the amino terminus of A β , indicating that β -secretase cleavage occurs in the secretory pathway as

well. This is confirmed by the detection of intracellular A β and APP $_{S-\beta}$ shortly after pulse labeling (Perez *et al.*, 1996).

Analysis of a rare APP mutation, the so-called "Swedish" mutation, has led to a better understanding of β -secretase cleavage and provided the first direct evidence for a role of enhanced A β production in AD. A double substitution of the two amino acids at positions P1 and P2 relative to the β -secretase cleavage site leads to early onset FAD in a Swedish family (Mullan *et al.*, 1992). The patients suffer from typical AD with a mean age of disease onset of ~55 years (Mullan *et al.*, 1992). Interestingly, in cells transfected with APP, this mutation causes a 5–8-fold increase in A β secretion (Citron *et al.*, 1992, Cai *et al.*, 1993) and a concomitant reduction in p3 secretion, compared to wild-type transfected cells (Citron *et al.*, 1992). The increase in A β secretion is also detected in primary fibroblasts (Citron *et al.*, 1994) and in plasma (Scheuner *et al.*, 1996) of both symptomatic and presymptomatic carriers of the Swedish APP mutation. This data provided the first direct link between a FAD genotype and the clinicopathological phenotype of increased A β deposition. The original study suggested that the Swedish mutation operates primarily to increase A β generation in the secretory pathway (Citron *et al.*, 1992). Indeed, it was subsequently shown that β -secretase cleavage of the Swedish mutant APP occurs in Golgi-derived secretory vesicles in the same compartment as α -secretase cleavage, explaining the simultaneous increase in A β and the reduction in p3 secretion (Haass *et al.*, 1995b, Thinakaran *et al.*, 1996). However, β -secretase cleavage of Swedish APP appears to be increased in the endocytic pathway as well (Perez *et al.*, 1996). Surprisingly, β -secretase cleavage has recently been shown to occur in the ER in NT2N neuronal cells (Chyung *et al.*, 1997). Therefore, more studies are needed before final conclusions about the cellular sites of the principal β -secretase cleavage can be made. Because the Swedish double substitution is at the P1 and P2 positions relative to the normal β -secretase cleavage site, it appeared quite reasonable to assume that it creates a better substrate for β -secretase, which would imply that β -secretase has at least some sequence specificity. A detailed mutagenesis study was therefore carried out to address the sequence specificity of β -secretase (Citron *et al.*, 1995). Using amino acid deletion and insertion constructs, it was demonstrated that β -secretase did not exhibit a distance-from-membrane specificity. Rather, mutations to the amino acid sequence around the cleavage site revealed that β -secretase is surprisingly sequence specific. At the P1 position A β production was maintained only by substituting the wild-type Met-1 with other bulky hydrophobic residues such as Tyr and Phe and upregulated by substitution with Leu, as occurs in the Swedish double mutation. At the P1' position, only an Asp $\rightarrow$ Glu substitution allowed the production of A β to occur. Furthermore, substitutions at P3 and P2' also caused significant decreases in A β production. Importantly, the sequence specificity appears to be similar in 293 kidney cells and in SK-N-SH human neuroblastoma cells, suggesting that the results are not only true for non-neural cells. Only membrane associated APP was found to undergo β -secretase cleavage, suggesting that APP needs to be membrane bound to be recognized by β -secretase (Citron *et al.*, 1995). However, the question whether β -secretase itself is membrane associated or not has not yet been answered.

As in the case of α -secretase, a closer look at β -secretase cleavage has suggested some raggedness of the N-terminal cleavage, with A β generated from wild-type APP starting primarily at Asp 1 but also at Val-3 and Glu 11 (Haass *et al.*, 1992b). The same wild-type APP gives rise to A β starting solely at Asp 1 in human primary skin fibroblasts (Citron *et al.*, 1994), while it starts predominantly at other positions in MDCK cells (Haass *et al.*, 1995a). Furthermore, treatment of cells with AEBSF differentially

affects the Asp1 vs. other forms of A β (Citron *et al.*, 1996a). Collectively, these data suggest the existence of several β -secretases which cleave at or near the Asp1 position of A β . This conclusion immediately raises questions about the relevance of observations in peripheral cell lines for processing in brain, particularly when it comes to quantitation of A β species. However, data on brain APP metabolism are now beginning to emerge from transgenic modeling. For example, a recent study has shown that the β -secretase processing of APP in transgenic mice is efficient in neurons but inefficient in astrocytes, suggesting that neurons are the major source for A β in the brain. This conclusion was based on the observation that the same APP transgene shows a much higher ratio of APP_s- β secretion to full-length (membrane bound) APP when expressed in neurons than in astrocytes (Zhao *et al.*, 1996). However, one has to bear in mind that the transgene in this study contained the Swedish mutation, and the possibility that the Swedish mutant molecule is cleaved by secretases distinct from or in addition to the "normal" β -secretase has not been ruled out. In this regard, the compound Bafilomycin A1 differentially affects β -secretase cleavage of wild-type and mutant APP (Knops *et al.*, 1995, Haass *et al.*, 1995b).

γ -secretase cleavage

A second proteolytic event, this one occurring within the A β sequence, is necessary to release A β (Figure 1). The enzyme(s) involved in this cleavage are even more elusive than β -secretase, because as we understand the transmembrane region of APP, this cleavage would have to occur within the membrane. Only one other similar cleavage has been reported. The SREBP proteins, involved in cholesterol metabolism, undergo two cleavages, the second of which apparently happens within the membrane bilayer (Brown and Goldstein, 1997). It appears quite clear that γ -secretase cleavage happens only after α - or β -secretase cleavage has occurred. While a secreted form of APPs terminating at the β -secretase site has been identified (Seubert *et al.*, 1993), no equivalent form ending at the C-terminus of A β has been confirmed. Instead, evidence has accumulated to suggest that α - and β -secretase cleavages each leave CTFs which then serve as the immediate precursors for γ -secretase cleavage. First, it was shown that a 12 kDa C-terminal fragment is detectable in cells transfected with APP (Shoji *et al.*, 1992), that its amount is increased in cells transfected with the Swedish mutant APP and that this fragment starts at Asp 1 (Cheung *et al.*, 1994). The amount of this fragment is decreased in cells in which A β production is blocked by substitutions around the β -secretase cleavage site, and substitutions which shift the β -secretase cleavage site shift the size of the CTF, as expected (Citron *et al.*, 1995). Finally, a reversible inhibitor of γ -secretase cleavage causes an increase in the amount of the 12 kDa fragment. Upon inhibitor washout, the amount of 12 kDa fragment decreases and A β production increases in a manner consistent with a precursor-product relationship (Higaki *et al.*, 1995). It therefore appears that γ -secretase can only cleave substrates which have already undergone the α - or β -secretase cleavage. Here, we hypothesize that a conformational change in the C-terminal portion of the APP molecule follows upon α - or γ -secretase cleavage. This postulated conformational change must make the CTF region more accessible to γ -secretase. At the same time, the conformation of the 12 kDa CTF may no longer allow it to be accessible to α -secretase. This could explain why expression of the last 100 amino acids of APP (i.e., the 12 kDa CTF) leads to the production of A β but not p3 (Dyrks *et al.*, 1993).

Mutations at codon 717 of APP₇₇₀ (position 46 from the A β start) which cause early onset FAD have been described (Goate *et al.*, 1991; Murrell *et al.*, 1991; Chartier-Harlin *et al.*, 1991). These mutations are close to the C-terminus of A β , suggesting that they might influence γ BETA-secretase cleavage. However, the mutations did not appear to change the overall amount of secreted A β (Cai *et al.*, 1993). In this regard, the exact cleavage site of γ -secretase has been more difficult to analyze than that of β -secretase, because the small CTF which should remain after γ -secretase cleavage has not been stable in sufficient quantities for radiosequencing. Mass spectrometry has shown that cultured cells secrete primarily A β 1–40 and lower amounts of C-terminally truncated species, but also secrete A β 1–42 (Dovey *et al.*, 1993; Wang *et al.*, 1996). The latter species has become the focus of intense research, as evidence has accumulated that A β 1–42 plays the key role in the process of plaque formation (see also the chapter by Younkin). This evidence includes: (i) *In vitro* data demonstrate that A β 1–42 accelerates the formation of fibrils by a nucleation dependent mechanism (Jarrett *et al.*, 1993). (ii) While accounting for only 10% of total A β secreted from cells (the vast majority is A β 1–40) (Dovey *et al.*, 1993; Asami-Odaka *et al.*, 1995), A β 1–42 is the major plaque component (Roher *et al.*, 1993; Iwatsubo *et al.*, 1994). Based on these findings, Suzuki *et al.* (1994) revisited the APP717 mutations looking for specific increases in A β 1–42 secretion. Indeed, while the amount of total secreted A β was not significantly elevated, the amount of A β 1–42 was 1.5–to 1.9 fold increased. Thus, an APP mutation can differentially influence the generation of A β 1–40 and A β 1–42, and the increase in A β 1–42 is sufficient to cause early onset FAD.

Even before the presenilin genes were cloned, experiments were started to address potential effects of mutations in the chromosome 14 FAD locus on APP processing by examining primary skin fibroblasts and the plasma of mutation carriers. Similar to the APP717 mutants, a selective, approximately two-fold increase in A β 1–42 production was observed in the presenilin subjects (Scheuner, 1996). These initial findings were confirmed and extended in transgenic mice and transfected cell lines, once the cloned presenilin genes had become available (Duff *et al.*, 1996; Borchelt *et al.*, 1996; Citron *et al.*, 1997). It is currently not understood exactly how a mutation in the presenilin genes causes increased A β 1–42 production *in trans*, but it is interesting to note that again only one of the two C-terminal cleavages is specifically elevated.

Very recently several studies have provided evidence for the generation of A β within the ER. This intracellular A β is primarily A β 1–42, in striking contrast to the secreted A β (Tienari *et al.*, 1997; Wild-Bode *et al.*, 1997; Cook *et al.*, 1997; Hartmann *et al.*, 1997; see also the chapter by Cook and Lee). The role of the A β 1–42 found in the ER is not currently understood. Does it get secreted or is it an irrelevant derivative that is degraded or does it kill cells from the inside? Whatever the role of intracellular A β 1–42 may be, its existence adds to the circumstantial evidence suggesting that A β 1–40 and A β 1–42 may be generated by different γ -secretases. The strongest evidence for this idea comes from studies using compounds that had originally been suggested as general γ -secretase inhibitors (Higaki *et al.*, 1995; Klafki *et al.*, 1995). In a pulse-chase paradigm, these compounds, which were initially found to inhibit the majority of A β secretion by directly or indirectly blocking γ -secretase, do in fact primarily affect γ -40 cleavage (Citron *et al.*, 1996b; Klafki *et al.*, 1996).

The identity of the γ -secretases is still unclear. It had been hypothesized that cathepsin D may be γ -secretase, however a careful *in vitro* study (Higaki *et al.*, 1996) and a cathepsin D knockout mouse which still produces A β 1–40 and A β 1–42 (Saftig *et al.*, 1996) provide strong evidence against that

idea. The sequence specificity of γ -secretase has been addressed in part (Tischer and Cordell, 1996). It was shown that substitution of negatively charged residues across positions 40–46 but not 48–50 precluded the formation of A β and APP maturation. These data suggest that the membrane boundary is at position 46/47. Deletions within the carboxyl terminal domain, including 4 residues spanning positions 39–42 of A β , still resulted in the formation of A β -like peptides. Substitution of residues 38–47 or 39–56 of A β with a transmembrane sequence from another protein still yielded a 4 kDa A β like peptide. From these data it was concluded that γ -secretase shows relaxed residue specificity (Tischer and Cordell, 1996). However, the observed total A β was not further analyzed for its percentage of A β 1–40, A β 1–42 or other truncated or elongated species. This makes the interpretation of these data more difficult. For example, it cannot yet be stated whether the A β 1–42 cleavage was affected by the various mutations.

CONCLUSION

The last few years have brought enormous progress in our understanding of APP processing. The subcellular localization of the various cleavage events and properties of the enzymes involved have been characterized. Various inhibitors of A β production have been developed and will soon be tested in clinical trials. The seminal role of A β 1–42—at least in early onset—AD has become generally accepted, particularly since it was found that the most malignant early onset FAD mutations (in the presenilin genes) act *in trans* to increase its production. However, 10 years after cloning of the APP molecule, no reports on the definite identification of any of the secretases have emerged. Thus, positive identification of α -, β -, and γ -secretases still remains a major challenge for the field.

REFERENCES

- Anderson, J.P., Esch, F.S., Keim, P.S., Sambamurti, K., Lieburg, I., and Robakis, N.K. (1991) Exact cleavage site of Alzheimer amyloid precursor in neuronal PC-12 cells. *Neurosci. Lett.*, **128**, 126–128.
- Arribas, J., Coodly, L., P.V., Kishimoto, T.K., Rose-John, S., and Massague, J. (1996) Diverse cell surface protein ectodomains are shed by a system sensitive to metalloprotease inhibitors. *J. Biol. Chem.*, **271**, 11376–11382.
- Arribas, J., and Massague, J. (1995) Transforming growth factor- α and β -amyloid precursor protein share a secretory mechanism. *J. Cell Biol.*, **128**, 433–441.
- Asami-Odaka, A., Ishibashi, Y., Kikuchi, T., Kitada, C., and Suzuki, N. (1995) Long amyloid β -protein secreted from wild-type human neuroblastoma IMR-32 cells. *Biochemistry*, **34**, 10272–10278.
- 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 1–42/1–40 ratio *in vitro* and *in vivo*. *Neuron*, **17**, 1005–1013.
- Boseman Roberts, S., Ripellino, J., Ingalls, K.M., Robakis, N.K., and Felsenstein, K.M. (1994) Nonamyloidogenic cleavage of the β -amyloid precursor protein by an integral membrane metalloendopeptidase. *J. Biol. Chem.*, **269**, 3111–3116.

- Brown, M.S., and Goldstein, J.L. (1997) The SREBP pathway: Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell*, **89**, 331–340.
- Busciglio, J., Gabuzda, D.H., Matsudaira, P., and Yankner, B.A. (1993) Generation of β -amyloid in the secretory pathway in neuronal and nonneuronal cells. *Proc. Natl. Acad. Sci. USA*, **90**, 2092–2096.
- Cai, X.-D., Golde, T.E., and Younkin, G.S. (1993) Release of excess amyloid β protein from a mutant amyloid β protein precursor. *Science*, **259**, 514–516.
- Caporaso, G.L., Gandy, S.E., Buxbaum, J.D., Ramabhadran, T.V., and Greengard, P. (1992) Protein phosphorylation regulates secretion of Alzheimer β /A4 amyloid precursor protein. *Proc. Natl. Acad. Sci. USA*, **89**, 3055–3059.
- Chartier-Harlin, M.-C., Crawford, F., Houlden, H., Warren, A., Hughes, D., Fidani, L., Goate, A., Rossor, M., Roques, P., Hardy, J., *et al.* (1991) Early-onset Alzheimer's disease caused by mutations at codon 717 of the β -amyloid precursor protein gene. *Nature*, **353**, 844–846.
- Chen, W.-J., Goldstein, J.L., and Brown, M.S. (1990) NPXY, a sequence often found in cytoplasmic tails, is required for coated-pit mediated internalization of the low density lipoprotein receptor. *J. Biol. Chem.*, **265**, 3116–3123.
- Cheung, T.T., Ghiso, J., Shoji, M., Cai, X., Golde, T., Gandy, S., Frangione, B., and Younkin, S. (1994) Characterization by radiosequencing of the carboxyl-terminal derivatavibes produced from normal and mutant amyloid β protein precursors. *Amyloid: Int. J. of Exp. Clin. Invest.*, **1**, 30–38.
- Chyung, A.S.C., Greenberg, B.D., Cook, D.G., Doms, R.W., and Lee, V.M.Y. (1997) Novel β -secretase cleavage of β -amyloid precursor protein in the endoplasmic reticulum/intermediate compartment of NT2N cells. *J. Cell Biol.*, **138**, 671–680.
- Citron, M., Diehl, T.S., Capell, A., Haass, C., Teplow, D.B., and Selkoe, D.J. (1996a) Inhibition of amyloid β -protein production in neural cells by the serine protease inhibitor AEBSE. *Neuron*, **17**, 171–179.
- Citron, M., Diehl, T.S., Gordon, G., Biere, A.L., Seubert, P., and Selkoe, D.J. (1996b) Evidence that A β 42 and A β 40 are generated from the β -amyloid precursor protein by different protease activities. *Proc. Natl. Acad. Sci. USA*, **93**, 13170–13175.
- Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A.Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., and Selkoe, D.J. (1992) Mutation of the β -amyloid precursor protein in familial Alzheimer's disease increases β -protein production. *Nature*, **360**, 672–674.
- Citron, M., Teplow, D.B., and Selkoe, D.J. (1995) Generation of amyloid β -protein from its precursor is sequence specific. *Neuron*, **14**, 661–670.
- Citron, M., Vigo-Pelfrey, C., Teplow, D.B., Miller, C., Schenk, D., Johnston, J., Winblad, B., Venizelos, N., Lannfelt, L., and Selkoe, D.J. (1994) Excessive production of amyloid β -protein by peripheral cells of symptomatic and presymptomatic patients carrying the Swedish familial Alzheimer's disease mutation. *Proc. Natl. Acad. Sci. USA*, **91**, 11993–11997.
- Citron, M., Westaway, D., Xia, W., Carlson, G., Diehl, T., Levesque, G., Johnson-Wood, K., Lee, M., Seubert, P., Davis, A., Kholodenka, D., Motter, R., Sherrington, R., Perry, B., Yao, H., Strome, R., Lieberburg, I., Rommens, J., Kim, S., Schenk, D., Fraser, P., St George-Hyslop, P., and Selkoe, D.J. (1997) Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid β -protein in both transfected cells and transgenic mice. *Nature Med.*, **3**, 67–72.
- Cook, D.G., Forman, M.S., Sung, J.C., Leight, S., Kolson, D.L., Iwatsubo, T., Lee, V.M.Y., and Doms, R.W. (1997) Alzheimer's A β (1–42) is generated in the endoplasmic reticulum/intermediate compartment of NT2N cells. *Nat Med.*, **3**, 1021–1023.

- De Strooper, B., Umans, L., Van Leuven, F., and Van Den Berghe, H. (1993) Study of the synthesis and secretion of normal and artificial mutants of murine amyloid precursor protein: Cleavage of APP occurs in a late compartment of the default secretion pathway. *J. Cell Biol.* **121**, 295–304.
- De Strooper, B., Van Leuven, F., and Van Den Berghe, H. (1992) $\alpha 2$ -macroglobulin and other proteinase inhibitors do not interfere with the secretion of amyloid precursor protein in mouse neuroblastoma cells. *FEBS Lett.*, **308**, 50–53.
- Dovey, H.F., Suomensaaari-Chrysler, S., Lieberburg, I., Sinha, S., and Keim, P.S. (1993) Cells with a familial Alzheimer's disease mutation produce authentic β -peptide. *Neuroreport*, **4**, 1039–1042.
- Duff, K., Eckman, C., Zehr, C., Yu, X., Prada, C.-M., Perez-tur, J., Mutton, M., Buee, L., Hairgaya, Y., Morgan, D., Gordon, M.N., Holcomb, L., Refolo, L., Zenk, B., Hardy, J., and Younkin, S. (1996) Increased amyloid A β 42(43) in brains of mice expressing mutant presenilin 1. *Nature*, **383**, 710–713.
- Dyrks, T., Dyrks, E., Mönning, U., Urmoneit, B., Turner, J., and Beyreuther, K. (1993) Generation of β A4 from the amyloid precursor and fragments thereof. *FEBS lett.*, **335**, 89–93.
- Esch, F.S., Keim, P.S., Beattie, E.C., Blacher, R.W., Culwell, A.R., Oltersdorf, T., McClure, D., and Ward, P.J. (1990) Cleavage of amyloid β -peptide during constitutive processing of its precursor. *Science*, **248**, 1122–1124.
- Estus, S., Golde, T.E., Kunishita, T., Blades, D., Lowery, D., Eisen, M., Usiak, M., Qu, X., Tabira, T., Greenberg, B.D., and Younkin, S.G. (1992) Potentially amyloidogenic carboxyl-terminal derivatives of the amyloid protein precursor. *Science*, **255**, 726–728.
- 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., James, L., Mant, R., Newton, P., Rooke, K., Roques, P., Talbot, C., Pericak-Vance, M., Roses, A., 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**, 704–706.
- Golde, T.E., Estus, S., Younkin, L.H., Selkoe, D.J., and Younkin, S.G. (1992) Processing of the amyloid protein precursor to potentially amyloidogenic carboxyl-terminal derivatives. *Science*, **255**, 728–730.
- Goldgaber, D., Lerman, M.I., McBride, O.W., Saffiotti, V., and Gajdusek, D.C. (1987) Characterization and chromosomal localization of a cDNA encoding brain amyloid of Alzheimer's disease. *Science*, **235**, 877–880.
- Gorevic, P., Goni, F., Pons-Estel, B., Alvarez, F., Peress, R., and Frangione, B. (1986) Isolation and partial characterization of neurofibrillary tangles and amyloid plaque cores in Alzheimer's disease: Immunohistological studies. *J. Neuropathol. Exp. Neurol.* **45**, 647–664.
- Haass, C., Hung, A.Y., Schlossmacher, M.G., Teplow, D.B., and Selkoe, D.J. (1993) β -amyloid peptide and a 3-kDa fragment are derived by distinct cellular mechanisms. *J. Biol. Chem.*, **268**, 3021–3024.
- Haass, C., Koo, E.H., Capell, A., Teplow, D.B., and Selkoe, D.J. (1995a) Polarized sorting of β -amyloid precursor protein and its proteolytic products in MDCK cells is regulated by two independent signals. *J. Cell Biol.*, **128**, 537–547.
- Haass, C., Koo, E.H., Mellon, A., Hung, A.Y., and Selkoe, D.J. (1992a) Targeting of cell-surface β -amyloid precursor protein to lysosomes: alternative processing into amyloid-bearing fragments. *Nature*, **357**, 500–503.
- Haass, C., Lemere, C.A., Capell, A., Citron, M., Seubert, P., Schenk, D., Lannfelt, L., and Selkoe, D.J. (1995b) Swedish mutation causes early-onset AD by β -secretase cleavage within the secretory pathway. *Nature Med.*, **1**, 1291–1296.

- Haass, C., Schlossmacher, M.G., Hung, A.Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B.L., Lieberburg, I., Koo, E.H., Schenk, D., Teplow, D.B., and Selkoe, D.J. (1992b) Amyloid β -peptide is produced by cultured cells during normal metabolism. *Nature*, **359**, 322–325.
- 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 β -40/42 amyloid peptides. *Nature Medicine*, 1016–1020.
- Higaki, J., Catalano, R., Guzzetta, A.W., Quon, D., Nave, J.F., Tarnus, C., D'Orchymont, H., and Cordell, B. (1996) Processing of β -amyloid precursor protein by cathepsin D. *J. Biol. Chem.*, **271**, 31885–31893.
- Higaki, J., Quon, D., Zhong, Z., and Cordell, B. (1995) Inhibition of β -amyloid formation identifies proteolytic precursors and subcellular site of catabolism. *Neuron*, **14**, 651–659.
- Hung, A.Y., and Selkoe, D.J. (1994) Selective ectodomain phosphorylation and regulated cleavage of β -amyloid precursor protein. *EMBO J*, **13**, 534–542.
- Iwatsubo, T., Odaka, A., Suzuki, N., Mizusawa, H., Nukina, H., and Ihara, Y. (1994) Visualization of A β 42(43) and A β 40 in senile plaques with end-specific A β monoclonals: evidence that an initially deposited species is A β 42(43). *Neuron*, **13**, 45–53.
- Jarrett, J.T., Berger, E.P., and Lansbury Jr, P.T. (1993) The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: Implications for the pathogenesis of Alzheimer's disease. *Biochemistry*, **32**, 4693–4697.
- Kang, J., Lemaire, H.-G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grzeschik, K.-H., Multhaup, G., Beyreuther, K., and Muller-Hill, B. (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature*, **325**, 733–736.
- Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S., and Ito, H. (1988) Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity. *Nature*, **331**, 530–532.
- Klafki, H.-W., Abramowski, D., Swoboda, R., Paganetti, P.A., and Staufenbiel, M. (1996) The carboxyl termini of β amyloid peptides 1–40 and 1–42 are generated by distinct gamma-secretase activities. *J. Biol. Chem.*, **271**, 28655–28659.
- Klafki, H.-W., Paganetti, P.A., Sommer, B., and Staufenbiel, M. (1995) Calpain inhibitor I decreases β A4 secretion from human embryonal kidney cells expressing β -amyloid precursor protein carrying the APP670/671 double mutation. *Neuroscience Letters*, **201**, 29–32.
- Knops, J., Suomensaaari, S., Lee, M., McConlogue, L., Seubert, P., and Sinha, S. (1995) Cell-type and amyloid precursor protein-type specific inhibition of a beta release by Bafilomycin A1, a selective inhibitor of vacuolar ATPases. *J. Biol. Chem.*, **270**, 2419–2422.
- Konig, G., Monning, U., Czech, C., Prior, R., Banati, R., Schreiter-Gasser, U., Bauer, J., Masters, C.L., and Beyreuther, K. (1992) Identification and differential expression of a novel alternative splice isoform of the β A4 amyloid precursor protein (APP) mRNA in leukocytes and brain microglial cells. *J. Biol. Chem.*, **267**, 10804–10909.
- Koo, E.H., and Squazzo, S. (1994) Evidence that production and release of amyloid β -protein involves the endocytic pathway. *J. Biol. Chem.*, **269**, 17386–17389.
- Kuentzel, S.L., Ali, S.M., Altman, R.A., Greenberg, B.D., and Raub, T.J. (1993) The Alzheimer β -amyloid protein precursor/protease nexin-II is cleaved by secretase in a trans-Golgi secretory compartment in human neuroglioma cells. *Biochem. J.*, **295**, 367–378.
- Lowery, D.E., Pasternack, J.M., Gonzalez-DeWhitt, P., Zurcher-Neely, H., Tomich, C.C., Altman, R.A., Fairbanks, M.B., Heinrikson, R.L., Younkin, S.G., and Greenberg, B.D. (1991) Alzheimer's amyloid precursor protein

- produced by recombinant baculovirus expression: proteolytic processing and protease inhibitory properties. *J. Biol. Chem.*, **266**, 19842–19850.
- Maruyama, K., Kametani, F., Usami, M., Yamao-Harigaya, W., and Tanaka, K. (1991) “Secretase” Alzheimer amyloid protein precursor secreting enzyme is not sequence-specific. *Biochem. Biophys. Res. Commun.*, **179**, 1670–1676.
- 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.
- Mullan, M., Crawford, F., Houlden, H., Axelman, K., Lilius, L., Winblad, B., and Lannfelt, L. (1992) A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of β -amyloid. *Nature Genet.*, **1**, 345–347.
- Murrell, J., Farlow, M., Ghetti, B., and Benson, M.D. (1991) A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. *Science*, **254**, 97–99.
- Nitsch, R.M., Slack, B.E., Wurtman, R.J., and Growdon, J.H. (1992) Release of Alzheimer amyloid precursor derivatives stimulated by activation of muscarinic acetylcholine receptors. *Science*, **258**, 304–307.
- Nordstedt, C., Gandy, S.E., Alafuzoff, I., Caporaso, G.L., Iverfeldt, K., Grebb, J.A., Winblad, B., and Greengard, P. (1991) Alzheimer β /A4-amyloid precursor protein in human brain: aging-associated increases in holoprotein and in a proteolytic fragment. *Proc. Natl. Acad. Sci. USA*, **88**, 8910–8914.
- Oltersdorf, T., Fritz, L.C., Schenk, D.B., Lieberburg, I., Johnson-Wood, K.L., Beattie, E.C., Ward, P.J., Blacher, R.W., Dovey, H.F., and Sinha, S. (1989) The secreted form of the Alzheimer's amyloid precursor protein with the Kunitz domain is protease nexin-II. *Nature*, **341**, 144–147.
- Oltersdorf, T., Ward, P.J., Henriksson, T., Beattie, E.C., Neve, R., Lieberburg, I., and Fritz, L.C. (1990) The Alzheimer amyloid precursor protein. Identification of a stable intermediate in the biosynthetic/degradative pathway. *J. Biol. Chem.*, **265**, 4492–4497.
- Palmert, M.R., Podlisny, M.B., Witker, D.S., Oltersdorf, T., Younkin, L.H., Selkoe, D.J., and Younkin, S.G. (1989) The β -amyloid precursor protein of Alzheimer disease has soluble derivatives found in human brain and cerebrospinal fluid. *Proc. Natl. Acad. Sci. USA*, **86**, 6338–6342.
- Perez, R.G., Squazzo, S.L., and Koo, E.H. (1996) Enhanced release of amyloid β -protein from Codon 670/671 “Swedish” mutant β -amyloid precursor protein occurs in both secretory and endocytic pathways. *J. Biol. Chem.*, **271**, 9100–9107.
- Ponte, P., Gonzalez-DeWhitt, P., Schilling, J., Miller, J., Hsu, D., Breenberg, B., Davis, K., Wallace, W., Lieberburg, I., Fuller, F., and Cordell, B. (1988) A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors. *Nature*, **331**, 525–527.
- Robakis, N.K., Ramakrishna, N., Wolfe, G., and Wisniewski, H.M. (1987) Molecular cloning and characterization of a cDNA encoding the cerebrovascular and the neuritic plaque amyloid peptides. *Proc. Natl. Acad. Sci. USA*, **84**, 4190–4194.
- Roher, A., Wolfe, D., Palutke, M., and KuKuruga, D. (1986) Purification, ultrastructure, and chemical analyses of Alzheimer's disease amyloid plaque core protein. *Proc. Natl. Acad. Sci. USA*, **83**, 2662–2666.
- Roher, A.E., Lowenson, J.D., Clarke, S., Woods, A.S., Cotter, R.J., Gowing, E., and Ball, M.J. (1993) β -amyloid-(1–42) is a major component of cerebrovascular amyloid deposits: implications for the pathology of Alzheimer's disease. *Proc. Natl. Acad. Sci. USA*, **90**, 10836–10840.
- Saftig, P., Peters, C., von Figura, K., Craessaerts, K., Van Leuven, F., and De Strooper, B. (1996) Amyloidogenic processing of human amyloid precursor protein in hippocampal neurons devoid of Cathepsin D. *J. Biol. Chem.*, **271**, 27241–27244.

- Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T.D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-Lahad, E., Viitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wasco, W., Lannfelt, L., Selkoe, D., and Younkin, S. (1996) Secreted amyloid β -protein similar to that in the senile plaques of Alzheimer's disease is increased *in vivo* by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nature Med.*, **2**, 864–870.
- Schubert, D., Jin, L.-W., Saitoh, T., and Cole, G. (1989) The regulation of amyloid β protein precursor secretion and its modulatory role in cell adhesion. *Neuron*, **3**, 689–694.
- Selkoe, D.J., Abraham, C.R., Podlisny, M.B., and Duffy, L.K. (1986) Isolation of low-molecular-weight proteins from amyloid plaque fibers in Alzheimer's disease. *J. Neurochem.*, **146**, 1820–1834.
- Selkoe, D.J., Podlisny, M.B., L., J.C., Vickers, E.A., Lee, G., Fritz, L.C., and Oltersdorf, T. (1988) β -amyloid precursor protein of Alzheimer disease occurs as 110–135 kilodalton membrane-associated proteins in neural and nonneural tissues. *Proc. Natl. Acad. Sci. USA*, **85**, 7341–7345.
- Seubert, P., Oltersdorf, T., Lee, M.G., Barbour, R., Blomquist, C., Davis, D.L., Bryant, K., Fritz, L.C., Galasko, D., Thal, L.J., Lieberburg, I., and Schenk, D.B. (1993) Secretion of β -amyloid precursor protein cleaved at the amino-terminus of the β -amyloid peptide. *Nature*, **361**, 260–263.
- Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., Sinha, S., Schlossmacher, M.G., Whaley, J., Swindlehurst, C., McCormack, R., Wolfert, R., Selkoe, D.J., Lieberburg, I., and Schenk, D. (1992) Isolation and quantitation of soluble Alzheimer's β -peptide from biological fluids. *Nature*, **359**, 325–327.
- Shoji, M., Golde, T.E., Ghiso, J., Cheung, T.T., Estus, S., Shaffer, L.M., Cai, X., McKay, D.M., Tintner, R., Frangione, B., and Younkin, S.G. (1992) Production of the Alzheimer amyloid β protein by normal proteolytic processing. *Science*, **258**, 126–129.
- Sisodia, S.S. (1992) β -amyloid precursor protein cleavage by a membrane-bound protease. *Proc. Natl. Acad. Sci. USA*, **89**, 6075–6079.
- Sisodia, S.S., Koo, E.H., Beyreuther, K., Unterbeck, A., and Price, D.L. (1990) Evidence that β -amyloid protein in Alzheimer's disease is not derived by normal processing. *Science*, **248**, 492–495.
- Suzuki, N., Cheung, T.T., Cai, X.-D., Odaka, A., Otvos Jr, L., Eckman, C., Golde, T.E., and Younkin, S.G. (1994) An increased percentage of long amyloid β protein secreted by familial amyloid β protein precursor (β APP717) mutants. *Science*, **264**, 1336–1340.
- Tamaoka, A., Kalaria, R.N., Lieberburg, I., and Selkoe, D.J. (1992) Identification of a stable fragment of the Alzheimer amyloid precursor containing the β -protein in brain microvessels. *Proc. Natl. Acad. Sci. USA*, **89**, 1345–1349.
- Tanzi, R.E., Gusella, J.F., Watkins, P.C., Bruns, G.A.B., St. George-Hyslop, P.H., 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.
- Tanzi, R.E., McClatchey, A.I., Lamperti, E.D., Villa-Komaroff, L., Gusella, J.F., and Neve, R.L. (1988) Protease inhibitor domain encoded by an amyloid protein precursor mRNA associated with Alzheimer's disease. *Nature*, **331**, 528–532.
- Thinakaran, G., Borchelt, D.R., Lee, M.K., Slunt, H.H., Spitzer, L., Kim, G., Rotovitsky, T., Davenport, F., Nordstedt, C., Seeger, M., Hardy, J., Levey, A.I., Gandy, S.E., Jenkins, N.A., Copeland, N.G., Price, D.L., and Sisodia, S.S. (1996) Endoproteolysis of presenilin 1 and accumulation of processed derivatives *in vivo*. *Neuron*, **17**, 181–190.
- 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 β -amyloid species are generated by distinct mechanisms in cultured hippocampal neurons. *Proc. Natl. Acad. Sci. USA*, **94**, 4125–4130.

- Tischer, E., and Cordell, B. (1996) β -amyloid precursor protein. Location of transmembrane domain and specificity of γ -secretase cleavage. *J. Biol. Chem.*, **271**, 21914–21919.
- van Nostrand, W.E., Wagner, S.L., Suzuki, M., Choi, B.H., Farrow, J.S., Geddes, J.W., Cotman, C.W., and Cunningham, D.D. (1989) Protease nexin-II, a potent anti-chymotrypsin, shows identity to amyloid β -protein precursor. *Nature*, **341**, 546–549.
- Walter, J., Hung, A.Y., Sisodia, S.S., Selkoe, D.J., and Haass, C. (1996) Phosphorylation of β -amyloid precursor protein at two distinct cellular locations. *Molec. Medicine*, 1896–1903.
- Wang, R., Meschia, J.F., Cotter, R.J., and Sisodia, S.S. (1991) Secretion of the β /A4 amyloid precursor protein. Identification of a cleavage site in cultured mammalian cells. *J. Biol. Chem.*, **266**, 16960–16964.
- Wang, R., Sweeney, D., Gandy, S.E., and Sisodia, S.S. (1996) The profile of soluble amyloid β protein in cultured cell media. Detection and quantification of amyloid β protein and variants by immunoprecipitation-mass spectrometry. *J. Biol. Chem.*, **271**, 31894–31902.
- Weidemann, A., Konig, G., Bunke, D., Fischer, P., Salbaum, J.M., Masters, C.L., and Beyreuther, K. (1989) Identification, biogenesis and localization of precursors of Alzheimer's disease A4 amyloid protein. *Cell*, **57**, 115–126.
- Wild-Bode, C., Yamazaki, T., Capell, A., Leimer, U., Steiner, H., Ihara, Y., and Haass, C. (1997) Intracellular generation and accumulation of amyloid β -peptide terminating at amino acid 42. *J. Biol. Chem.*, **272**, 16085–16088.
- Yamazaki, T., Koo, E.H., and Selkoe, D.J. (1996) Trafficking of cell-surface amyloid β -protein precursor. II. Endocytosis, recycling and lysosomal targeting detected by immunolocalization. *J. Cell Sci.*, **109**, 999–1008.
- Zhao, J., Paganini, L., Mucke, L., Gordon, M., Refolo, L., Carman, M., Sinha, S., Oltersdorf, T., Lieberburg, L., and McConlogue, L. (1996) β -secretase processing of the β -amyloid precursor protein in transgenic mice is efficient in neurons but inefficient in astrocytes. *J. Biol. Chem.*, **271**, 31407–31411.
- Zhong, Z., Higaki, J., Murakami, K., Wang, Y., Catalano, R., Quon, D., and Cordell, B. (1994) Secretion of β -amyloid precursor protein involves multiple cleavage sites. *J. Biol. Chem.*, **269**, 627–632.

6.

BIOSYNTHESIS OF APP AND A β : MULTIPLE PATHWAYS FOR THE GENERATION OF INTRACELLULAR A β

DAVID G.COOK¹, MARK S.FORMAN², ABRAHAM S.C.CHYUNG², ROBERT W.DOMS³ and VIRGINIA M.-Y.LEE²

¹*Veterans Affairs, Puget Sound Health Care System GRECC (182B), 1660 S.Columbian Way, Seattle, WA 98108*

²*Department of Pathology and Laboratory Medicine, third floor Maloney, HUP, Philadelphia, PA 19104*

³*Department of Pathology and Laboratory Medicine, University of Pennsylvania Medical Center, Abramson Research Center, Room 806, 34th and Civic Center Blvd., Philadelphia PA 19104*

INTRODUCTION

The capacity of the central nervous system to retain the experiences of an individual is a critical biological function. The significance of this function is no better illustrated than by considering the clinical consequences of Alzheimer's disease (AD), well-characterized for causing progressive and permanent disruption of memory in affected individuals (Selkoe, 1994). There are a wide array of neuropathological features of AD which include the development of neurofibrillary tangles (Goedert *et al.*, 1991; Lee and Trojanowski, 1992), neuronal loss (Cotman and Su, 1996; Gomez-Isla *et al.*, 1996) and disturbances in a number of neurotransmitter systems (Palmer, 1996). However, the histopathological hallmark of AD is the florid deposition of compact and diffuse senile plaques (Tagliavini *et al.*, 1988; Yamaguchi *et al.*, 1988; Wisniewski *et al.*, 1989). Amyloid β peptide (A β), the primary proteinaceous component of these plaques, ranges from 39–43 amino acids in length (Glenner and Wong 1984; Masters *et al.*, 1985). A β is derived from post-translational cleavage of the amyloid precursor protein (APP) (Goldgaber *et al.*, 1987; Tanzi *et al.*, 1987; Kang *et al.*, 1987; Robakis *et al.*,

1987). Alternative splicing of APP mRNAs can give rise to eight A β containing APP isoforms, of which APP 695, 751, and 770 are the three principal species (Figure 1). APP 751 differs from APP 695 by the inclusion of a 56 amino acid domain referred to as the Kunitz protease inhibitor domain (KPI) due to its homology to the Kunitz serine protease inhibitor family (Kang *et al.*, 1987; Kitaguchi *et al.*, 1988; Ponte *et al.*, 1988; Tanzi *et al.*, 1988). APP 770 contains the KPI domain, as well as a 19 amino acid sequence with homology to the MRC OX-2 antigen (Kitaguchi *et al.*, 1988; Weidemann *et al.*, 1989). Whether these domains influence the processing of APP has not been established.

APP is ubiquitously expressed, although the highest levels of expression occur in the brain and kidney (Goedert, 1987; Neve *et al.*, 1988; Golde *et al.*, 1990; Koo *et al.*, 1990). While the most abundant APP isoform is APP 751, neurons express APP 695 almost exclusively (Golde *et al.*, 1990; Kang and Muller-Hill, 1990; Arai *et al.*, 1991). APP 751 and APP 770 are expressed primarily in peripheral tissues. Glial cells also express significant levels of the KPI-containing isoforms. Thus, within the brain any of the major APP isoforms are, in principle, a source of A β . Currently, the function of APP is not well understood. However, it is thought that APP may play a role in neural development and neurite outgrowth (Small *et al.*, 1994), neuronal excitability (Furukawa *et al.*, 1996) and memory (Luo *et al.*, 1992; Muller *et al.*, 1994).

Structurally, APP is a type 1 integral membrane protein that contains both N- and O-linked carbohydrate chains (Figure 1) (Weidemann *et al.*, 1989; Kang *et al.*, 1987; Oltersdorf *et al.*, 1990; Knops *et al.*, 1993). The first 28 amino acids of the A β sequence are located in the extracellular domain, with the remaining 11–15 residues embedded in the transmembrane region (Figure 1). Thus, in order for A β to be generated from APP, at least two proteolytic cleavage events must occur. Finally, the cytoplasmic domain of APP contains an NPTY internalization motif which can cause the protein to be endocytosed following delivery to the cell surface (Golde *et al.*, 1992; Haass *et al.*, 1992a; Nordstedt *et al.*, 1993; Koo and Squazzo, 1994; Lai *et al.*, 1995; Perez *et al.*, 1996).

As APP transits the secretory pathway it undergoes a series of characteristic endoproteolytic cleavages mediated by a number of currently unidentified proteases referred to collectively as the α , β , and γ -secretases (Figure 1). APP can first be cleaved by β -secretase, leading to the generation of a soluble ectodomain fragment termed APP β and a C-terminal fragment containing the entire A β sequence. Subsequent cleavage by γ -secretase generates A β . By contrast, α -secretase cleaves APP within the A β sequence, precluding the generation of A β (Sisodia *et al.*, 1990). Instead, cleavage of APP by α -secretase generates a large, secreted ectodomain fragment (APP α) and a membrane anchored C-terminal fragment. Further cleavage of this C-terminal fragment by γ -secretase yields the p3 fragment (Figure 1).

The seemingly straight forward relationship between APP metabolism yielding secreted A β that eventually deposits in the senile plaques of AD brains belies a highly complex disease process with numerous distinct etiologies (Selkoe, 1997a). AD is a disease arising from multiple dysfunctions and is expressed both as early onset familial cases and late onset forms. While most cases of late onset AD have no clear genetic component, in some individuals it is associated with inheritance of specific alleles of apolipoprotein E (Roses, 1995) and more recently with mutations in mitochondrial cytochrome c oxidase genes (Davis *et al.*, 1997). With respect to familial AD (FAD) specific mutations in genes located on chromosomes 21 (APP), 14 (presenilin 1), and 1 (presenilin 2) have been identified which cause early onset forms of FAD (discussed below) (Goate *et al.*, 1991; Chartier-Harlin *et al.*, 1991; Murrell *et*

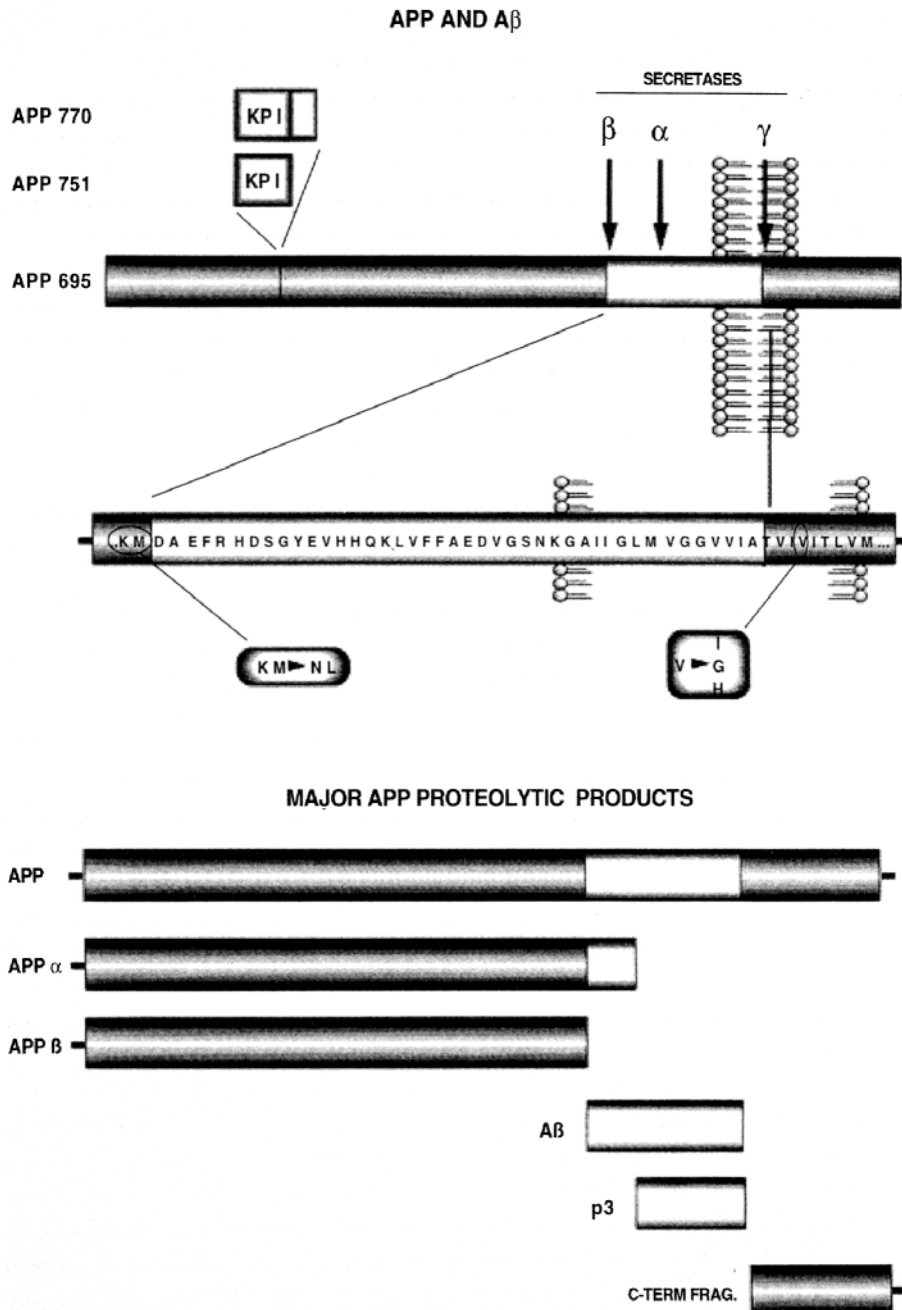


Figure 1. Structure of APP: Three primary isoforms of APP: APP 695; APP 751 containing a 56 amino acid insert with homology to the Kunitz protease inhibitor (KPI) family; and APP 770 containing both the KPI domain and an additional 19 amino acid sequence insertion with homology to the MRC OX-2 antigen. Indicated familial AD (FAD) mutations flank the N- and C-terminal regions of A β . APP undergoes several post-translational secretase cleavages (α , β , γ) that yield several proteolytic APP-derived products including APP β , APP α , A β , p3, and C-terminal fragments.

al., 1991; Mullan *et al.*, 1992; Sherrington *et al.*, 1995; Rogaev *et al.*, 1995; Levy-Lahad *et al.*, 1995a,b). In some instances, FAD patients can develop symptoms as early as the fourth decade of life (Alzheimer's Disease Collaborative Group, 1995).

Despite the heterogeneity in the causes of AD, a number of lines of evidence suggest that APP and A β play a critical role in the cascade of events causing AD. Evidence favoring this view comes from studies of Down's syndrome patients who develop AD, presumably by virtue of having an extra copy of the APP gene on chromosome 21. Significantly, these individuals begin to develop A β plaques years prior to the manifestation of other pathological markers of the disease, arguing that the development of plaques may be an initiating factor in the cascade of events that give rise to AD in Down's patients (Giaccone *et al.*, 1989; Mann *et al.*, 1990; Selkoe, 1994). Some of the strongest support for the direct role of A β biosynthesis in AD comes from the fact that a number of cases of FAD are associated with point mutations within the APP gene that lead to amino acid substitutions at either end of the A β sequence (Figure 1). Mutations which change amino acids 670 and 671 from Lys-Met to Asn-Lys cause a dramatic increase in the generation of A β (Citron *et al.*, 1992; Cai *et al.*, 1993; Citron *et al.*, 1994), while mutations near the carboxyl-terminus of A β cause a shift in the processing of APP that favors the production of longer forms of A β ending in Ala-42, termed A β (42) (Suzuki *et al.*, 1994). A β (42) may play a critical role in AD because this form of A β is initially deposited in senile plaques of AD patients (Iwatsubo, 1994), individuals with Down's syndrome (Iwatsubo *et al.*, 1995), and in normal brains (Fukumoto *et al.*, 1996). A β (42) is also more prone to form insoluble A β fibrils than A β (40) *in vitro* (Jarrett *et al.*, 1993; Jarrett and Lansbury, 1993). Additional *in vivo* evidence that alterations in APP processing can lead to AD pathology comes from mice transgenic for FAD-associated APP mutations. These mice exhibit many of the pathological features of AD and have been reported to show learning and memory deficits corresponding with the development of A β plaques in the central nervous system (Games *et al.*, 1995; Hsiao *et al.*, 1996; Johnson-Wood *et al.*, 1997).

Recent studies with the presenilins have shown that mutations in PS1 and PS2 may also alter APP processing. Specifically, mutations in both PS1 and PS2 alter the processing of APP in a manner that increases the generation of A β (42) relative to A β (40) (Scheuner *et al.*, 1996; Duff *et al.*, 1996; Lemere *et al.*, 1996; Tomita *et al.*, 1996). Thus it appears that even for FAD caused by mutations in genes other than APP, a common theme in AD pathogenesis may involve alterations in normal APP processing that directly or indirectly cause increased production of A β , with the generation of A β (42) being particularly pathogenic. Therefore, understanding the mechanisms of APP processing is a critical step in understanding the AD disease process. Accordingly, a great deal of attention has been paid to this issue and a number of significant APP processing pathways contributing to the generation of A β have been identified and characterized. Much of this work has been reviewed elsewhere (Selkoe, 1994; 1997b; Golde and Younkin, 1995; Checler, 1995). The purpose of this chapter is to review a number of recent reports identifying a novel pathway in the endoplasmic reticulum/intermediate compartment (ER/IC) responsible for generating A β (42) and to place these new findings in context with the current understanding of the mechanisms governing APP and A β metabolism.

MULTIPLE PATHWAYS FOR THE GENERATION OF INTRACELLULAR A β

The findings outlined above collectively argue that altered processing of A β may be a critical step in the complex processes leading to AD. An intensive effort to elucidate the mechanisms governing the metabolism of APP has revealed that there are multiple path-ways that process APP. In non-neuronal cells, the dominant pathway involves the α -secretase cleavage of APP between amino acids 16 and 17 of the A β peptide domain (Sisodia *et al.*, 1990; Esch *et al.*, 1990). Subsequent cleavage of the residual C-terminal fragment by γ -secretase generates the P3 fragment (Figure 1), which has been detected in senile plaques (Gowing *et al.*, 1994). Most evidence indicates that α -secretase activity occurs quite late in the secretory pathway or at the cell surface (Sambamurti *et al.*, 1992; Sisodia, 1992; Kuentzel *et al.*, 1993; DeStrooper *et al.*, 1993). Unlike most endoproteolytic cleavages, α -secretase has little sequence specificity. Properties such as distance from the membrane surface and secondary structure appear to regulate its sites of activity (Sisodia, 1992). The apparent lack of substrate specificity by α -secretase suggests that its action may reflect the combined action of multiple secretases (Zhong *et al.*, 1994). Like non-neuronal cells, neurons also exhibit α -secretase activity. However, neurons do not express intracellular p3 and the amount of p3 found in the medium of neuronal cells is low compared to non-neuronal cells (Hung *et al.*, 1992; Chyung *et al.*, 1997; Tienari *et al.*, 1997).

Whereas α -secretase cleaves APP such that the A β sequence is truncated, the β -secretase cleaves APP to produce C-terminal fragments that contain full-length A β sequences. The precise site of the β -secretase activity is both sequence and cell type specific (Citron *et al.*, 1995). The fact that β -secretases from different cells cleave APP at slightly different sites suggests that there are multiple β -secretases or multiple sites where cleavage occurs (Haass *et al.*, 1992b; Haass *et al.*, 1994; Citron *et al.*, 1995). At present, at least three β -secretase pathways that mediate the generation of A β have been described: an endosomal/ lysosomal pathway; a late β -secretory pathway; and a recently discovered endoplasmic reticulum/intermediate compartment (ER/IC) pathway (Figure 2).

Endosomal/Lysosomal Pathway

Full length APP escaping α -secretase activity can undergo internalization and be recovered in clatharin-coated vesicles (Nordstedt *et al.*, 1993; Koo and Squazzo, 1994; Lai *et al.*, 1995). In neurons this APP is sorted to an early endosomal compartment and transported back to the somato-dendritic compartments of the cell (Marquez-Sterling *et al.*, 1997). That these acidic endosomal/lysosomal compartments could serve as sites for the generation of A β is supported by the findings that agents altering intracellular pH such as NH₄ Cl and chloroquine reduce A β secretion (Shoji *et al.*, 1992; Haass *et al.*, 1993). However, A β has not been recovered from endosomes or lysosomes (Haass *et al.*, 1993) and other recent studies indicate that it may not be a site of A β production (Paraus *et al.*, 1997).

Late β -Secretory Pathway

In addition to the endosomal/lysosomal pathway there is mounting evidence supporting the existence of an alternative β -secretory pathway in the late Golgi/trans Golgi network (TGN) and in Golgi-derived vesicles (Haass *et al.*, 1995; Xu *et al.*, 1997). In this pathway it is possible that A β is generated as early

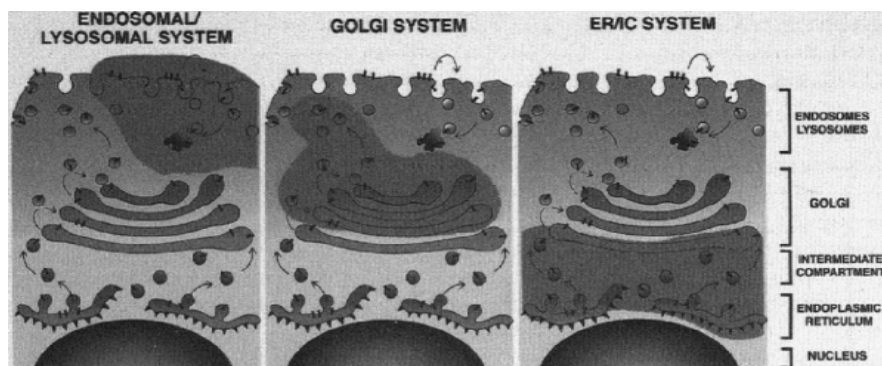


Figure 2. Three β -Secretory APP and $A\beta$ Processing Pathways: $A\beta$ is generated intracellularly by three primary β -secretory pathways: an endosomal/lysosomal pathway occurring after full length APP is reinternalized from the cell surface; a Golgi and Golgi-derived vesicle pathway where $A\beta$ is produced in late Golgi structures (trans Golgi and trans Golgi network); and a newly discovered endoplasmic reticulum/intermediate compartment (ER/IC) pathway. Specified intracellular domains are shaded. APP in the secretory pathway is schematized as small dark bars. In the ER/IC pathway $A\beta(42)$ exclusively is generated, while $A\beta(40)$ is predominately produced in the endosomal/lysosomal and late Golgi pathways.

as the medial Golgi (Thinakaran *et al.*, 1996) since pulse-chase studies showed that $APP\beta$ is first detected concurrent with acquisition of endoglycosidase H resistance (an indication that proteins have entered the medial Golgi). Moreover, the activity of the γ -secretase has been localized to the same region of the cell by analyzing the action of a peptide aldehyde γ -secretase inhibitor (Higaki *et al.*, 1995; Citron *et al.*, 1996). Taken together, these findings argue that the more distal intracellular organelles of the protein secretory system are significant sites for the generation of $A\beta$. This view is further supported by the findings that brefeldin A (BFA), a compound that blocks transport of secretory proteins from the ER/IC (Doms *et al.*, 1989; LippincottSchwartz *et al.*, 1989), inhibits the secretion of $A\beta$ (Haass *et al.*, 1993; Busciglio *et al.*, 1993; Higaki *et al.*, 1995; Haass *et al.*, 1995; Martin *et al.*, 1995).

ER/IC Pathway

In view of the results outlined above it has come as a surprise to find that there is a third intracellular amyloidogenic pathway for generating $A\beta$ that is active early in the secretory pathway at the level of the ER/IC (Chyung *et al.*, 1997; Cook *et al.*, 1997; Hartmann *et al.*, 1997; Wild-Bode *et al.*, 1997). This pathway had not been previously identified even in cells overexpressing APP because until recently intracellular $A\beta$ had been detectable only in post-mitotic neurons (Wertkin *et al.*, 1993; Turner *et al.*, 1996). Consequently, much of the previous work aimed at determining the intracellular sites of $A\beta$ biosynthesis have, of necessity, inferred intracellular mechanisms based upon measurements of $A\beta$ secreted into the medium.

Intracellular $A\beta$ was first detected by Wertkin and colleagues (1993) examining NT2N neurons. Further characterization showed that NT2N neurons endogenously produce both $A\beta(40)$ and $A\beta(42)$

intracellularly (Turner *et al.*, 1996). In addition, low levels of intra cellular A β have been detected in the neuroblastoma SH-SY5Y cell line (Fuller *et al.*, 1995). The capacity to measure intracellular A β in a human neuronal cell system, combined with the ability to overexpress foreign genes in post-mitotic cells using Semliki forest virus (SFV) vectors (Liljestrom and Garoff, 1991) has made it possible to examine the question of which intracellular organelles contribute to the generation of A β in far greater detail than previously possible (Simons *et al.*, 1996; Tienari *et al.*, 1997; Cook *et al.*, 1997; Hartmann *et al.*, 1997). For example, treatment of NT2N neurons overexpressing wildtype APP with BFA blocks A β secretion but not its intracellular production (Chyung *et al.*, 1997; Cook *et al.*, 1997; Figure 3A,B). Surprisingly, BFA treatment was shown to selectively inhibit production of intracellular A β (1–40), while A β (1–42) was largely unaffected (Figure 3C,D) (Cook *et al.*, 1997). These findings are in accord with the data of Wild-Bode and colleagues (1997) who found that the intracellular post-nuclear supernatant from kidney 293 cells contained both A β (1–42) and A β (X–42), but no A β (40) species. Both BFA treatment and expression of the FAD APP mutant Val to Gly 717, previously shown to increase A β (42) secretion (Suzuki *et al.*, 1994), elevated intracellular levels of A β (42) (Wild-Bode *et al.*, 1997).

These studies clearly demonstrated that A β (42) is produced in the secretory pathway, perhaps in the ER/IC. However, BFA blocks transport of all proteins from the ER/IC, and so might also retain the β and γ -secretases. Under such circumstances these secretases might exhibit aberrant cleavage activity. This concern was addressed by retaining APP in the ER/ IC by including a di-lysine ER retrieval signal at the C-terminus of wild-type APP (Cook *et al.*, 1997). As a result, APP was specifically retained in the ER/IC and, as with BFA, intracellular A β was reduced but not abolished (Figure 4A,B). This reduction was due to the loss of A β (40), while intracellular A β (42) levels were comparable to those generated from cells expressing non-ER retained APP (Figure 4C,D). To further support that A β (42) is indeed produced in the ER/IC, we demonstrated that the large ectodomain of APP generated by β -secretase cleavage (APP β) can also be detected under conditions where APP is retained in the ER/IC (Chyung *et al.*, 1997). These results are consistent with the observation that potentially amyloidogenic C-terminal fragments accumulate in the presence of BFA (Gabuzda *et al.*, 1994). Other support for the idea that A β (42) is generated in the ER/IC has come from the ultrastructural studies of A β expression in neurons in which Hartmann *et al.* (1997) demonstrated the ER localization of A β (42) using immuno-electron microscopy. In contrast, A β (40) immuno-reactivity was found only in the (TGN). Interestingly, in non-neuronal cells both A β (40) and A β (42) immuno-reactivity were seen only near the plasma membrane (Hartmann *et al.*, 1997), perhaps helping to account for why intracellular A β (42) has been detected only recently and suggesting that a greater fraction of APP is processed by the ER/IC in neurons than in non-neuronal cells.

RELATIONSHIP BETWEEN INTRACELLULAR AND SECRETED A β

The discovery that A β (42) is largely produced in the ER/IC suggests that there may be at least two different pools of A β : a secretable pool composed primarily of A β (40); and a pool of intracellular A β , composed primarily of A β (42). This view is supported by the findings that where intracellular A β (40) and A β (42) have been individually analyzed, the ratio A β (42)/A β (40) has been greater in the cell than in the medium (Turner *et al.*, 1996; Klafki *et al.*, 1996; Wilde-Bode *et al.*, 1997; Tienari *et al.*,

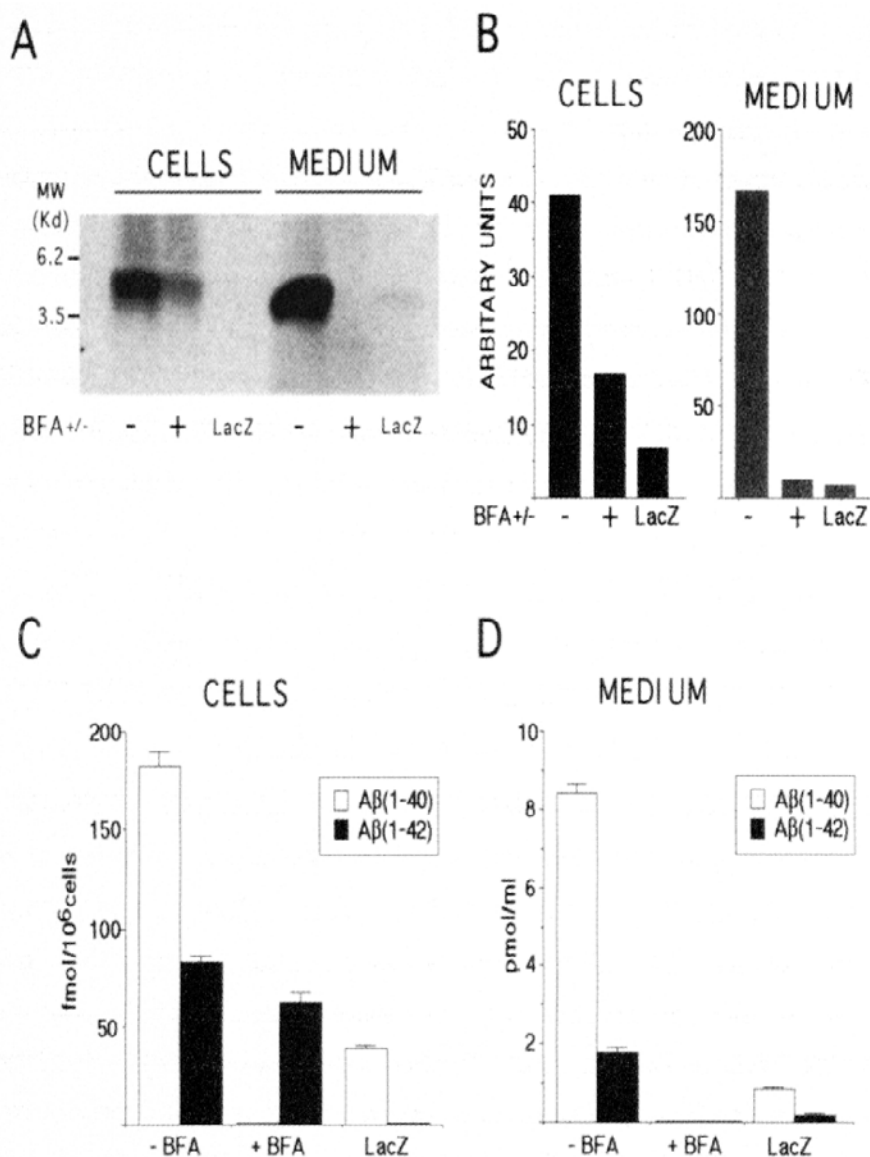


Figure 3. Effects of BFA on A β Expression in NT2N Neurons: (A) NT2N neurons infected with a Semliki forest virus (SFV) vector expressing wild-type APP 695 were treated +/- Brefeldin A (BFA) (10 μ g/ml) for 1 hour prior to and during an 8 hour metabolic labeling with [^{35} S] methionine. NT2N neurons infected with SFV-LacZ (without BFA) served as controls. A β was immunoprecipitated from cell lysates and medium with the monoclonal antibody BAN-50 (6.5 μ g/ml) and resolved on a tris-tricine gel system. Treatment with BFA reduced but did not block intracellular A β . (B) Quantification of phosphorimager signals shown in (A). (C and D) NT2N neurons infected with SFV wild type APP 695 were incubated overnight +/- BFA (10 μ g/ml), after which cell lysates (C) and medium (D) were analyzed by ELISA (Suzuki *et al.*, 1994) to quantify A β (1-40) and A β (1-42) levels. Intracellular A β (1-40), but not intracellular A β (1-42) was blocked by BFA treatment. For (C) and (D) error bars show standard error of the mean (S.E.M.) for 3 independent sample wells (approximately 2×10^6 cells per well) within one experiment. Comparable results were obtained in 7 out of 7 independent experiments (Cook *et al.*, 1997).

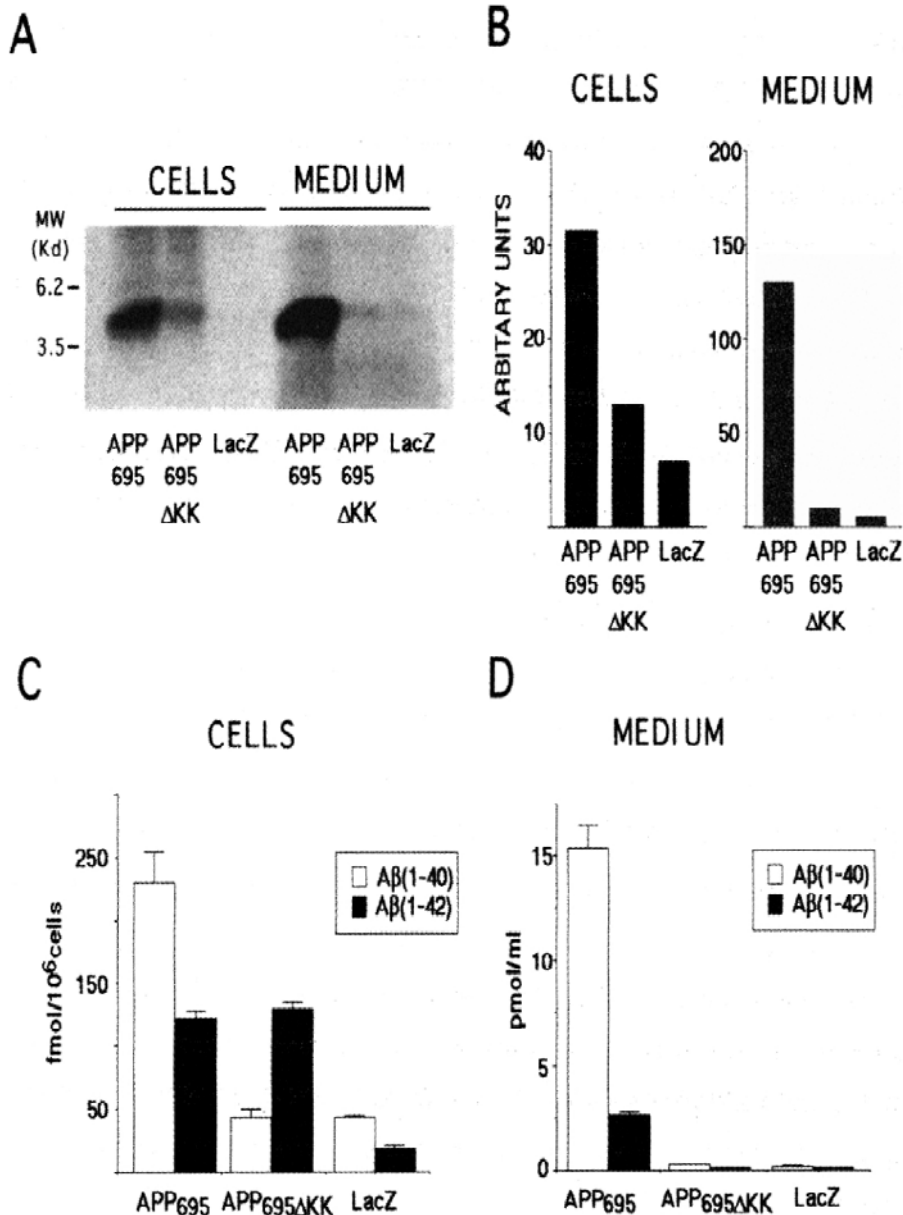


Figure 4. Effects of Retaining APP in the ER/IC on the Generation of A β : (A) NT2N neurons infected with SFV-wild type APP 695 or wild type APP 695 with a di-lysine (Δ KK) ER retention signal placed at the third and fourth amino acids from the extreme C-terminus of APP were metabolically labeled overnight, followed by immunoprecipitation of A β from cell lysates and medium with BAN-50 (6.5 μ g/ml). LacZ expressing cells served as controls. Retention of APP in the ER/IC reduced, but did not block generation of intracellular A β . (B) Quantification of phosphoimager signals shown (A). Note difference in scale between cells and medium. Cell lysates (C) and medium (D) were analyzed by ELISA to quantify levels of A β (1–40) and A β (1–42) as in Figure 3. As with BFA treatment (Figure 3) retention of APP in the ER/IC specifically blocked expression of A β (1–40) but not A β (1–42). Error bars show S.E.M. for 3 independent sample wells (approximately 2×10^6 cells per well) within one experiment. The same results were obtained in 4 out of 4 independent experiments (Cook *et al.*, 1997).

1997). Also, the APP 717 Δ I and Δ G FAD mutations, known to favor the production of A β (42) over A β (40) (Suzuki *et al.*, 1997), differentially enhance the levels of A β (42) inside the cell compared to the medium (Tienari *et al.*, 1997; Wild-Bode *et al.*, 1997). Consistent with their different fates, it appears that the readily secreted A β pool is generated in different subcellular organelles than the intracellular A β (42) pool. This interpretation is supported by the findings that NH₄Cl, which in non-neuronal cells reduces secretion of A β (Shoji *et al.*, 1992; Haass *et al.*, 1993), has no effect on intracellular A β (Tienari *et al.*, 1997). Similarly, expression of APP lacking a C-terminal endocytosis signal, previously shown to affect A β secretion (Koo and Squazzo, 1994; Lai *et al.*, 1995), had no clear impact on the production of intracellular A β (Tienari *et al.*, 1997). In addition, both monensin (which inhibits protein transport beyond the trans-Golgi) and treatment of cells at 20 degrees C (retards proteins transport from the trans-Golgi) fail to inhibit production of intracellular A β in cells over-expressing the Swedish Δ NL form of APP (Martin *et al.*, 1995). Taken together, these data argue for a model where A β (42) is primarily generated in the early secretory pathway (ER/ IC), while A β (40) is produced in later secretory compartments (late Golgi, trans-Golgi network, and the endosomal/lysosomal system).

PROCESSING OF WILD-TYPE AND MUTANT APP IN NEURONAL VERSUS NON-NEURONAL CELLS

Comparison of APP processing in a variety of cell types indicates that while the pathways leading to the production of A β appear to be conserved, the relative importance of each can vary considerably between neurons and non-neuronal cells. Perhaps the most fundamental cell type dependent difference in APP processing is that the α -secretory pathway is utilized preferentially over β -secretory pathways in non-neuronal cells (Esch *et al.*, 1990; Haass *et al.*, 1992a; Seubert *et al.*, 1993). Conversely, processing of APP by β -secretory pathways is more pronounced in neurons, perhaps explaining why constitutively produced, intracellular A β can be detected in these cells (Wertkin *et al.*, 1993; Turner *et al.*, 1996; Chyung *et al.*, 1997). However, overexpression of APP in a variety of non-neuronal cell types leads to detectable levels of intracellular A β , indicating that the intracellular pathways for A β production are conserved but differentially utilized depending on cell type (Wertkin *et al.*, 1993; Fuller, *et al.*, 1995; Perez *et al.*, 1996; Turner *et al.*, 1996; Tienari *et al.*, 1997; Cook *et al.*, 1997; Wild-Bode *et al.*, 1997; Hartmann *et al.*, 1997; Forman *et al.*, 1997). Furthermore, EM localization of A β (40) and A β (42) suggests that most of the intracellular A β (42) is generated near the plasma membrane in non-neuronal cells, while in neurons most is made in the ER (Hartmann, *et al.*, 1997). Thus, non-neuronal cells favor not only α -secretase over β -secretase processing, but they also favor β -secretase processing that occurs late over the A β (42)-specific ER/IC pathway which occurs early in the biosynthetic pathway.

Differential utilization of both α and β -secretase pathways by neuronal and non-neuronal cells may have implications for how FAD-associated APP mutants are processed. For example, non-neuronal cells can generate intracellular A β and APP β from the Swedish APP Δ NL mutation, suggesting that this mutation diverts APP into an otherwise non-utilized metabolic pathway that has similarities to the APP processing pathways found in neurons (Felsenstein *et al.*, 1994; Martin *et al.*, 1995; Haass *et al.*, 1995; Thinakaran *et al.*, 1996; Perez *et al.*, 1996; Forman *et al.*, 1997). Interestingly, expression of APP Δ NL in nonneuronal cells causes a 5–10 fold increase in the production of total A β (Citron *et al.*,

1992; Cai *et al.*, 1993; Suzuki *et al.*, 1994; Haass *et al.*, 1995; Perez *et al.*, 1996; Thinakaran *et al.*, 1996; Forman *et al.*, 1997), while in neurons the increase in A β caused by the Δ NL mutation is much less pronounced: only a two-fold increase as determined by immunoprecipitation (DeStrooper *et al.*, 1995) and a 0–35% increase as measured by quantitative A β capture ELISA (Forman *et al.*, 1997). This marked distinction in the processing of APP Δ NL in neurons compared to non-neuronal cells underscores the importance of cell type when evaluating APP processing, and is consistent with the hypothesis that neurons preferentially process APP by β -secretase pathways while nonneuronal cells favor α -secretase pathways for the processing of wt APP. The shift towards β -processing seen in non-neuronal cells following expression of APP Δ NL again indicates that the major processing pathways are conserved between cell types, but that they are differentially utilized.

ACTIVITY OF γ -SECRETASE

The existence of multiple, cell specific pathways for the generation of A β highlights the issue of whether or not there are multiple secretases acting at specific locations in the cell. Currently, neither β or γ -secretase have been identified. Thus, this issue cannot yet be fully addressed. However, analyses of APP processing using a number of protease inhibitors suggests that there might be multiple γ -secretases. In support of this idea, the calpain inhibitor MDL28170 has been shown to be a γ -secretase inhibitor that may involve the early endosomal compartment (Higaki *et al.*, 1995). Subsequent studies with MDL28170 showed that it was much more efficient at blocking γ -secretase activity generating A β (40) than A β (42) (Citron *et al.*, 1996). Three closely related peptide aldehyde protease inhibitors, calpain inhibitor I, calpeptin, and MG132 also differentially affected the production of A β (40) and A β (42) (Klafki *et al.*, 1996; Yamazaki *et al.*, 1997). However, depending upon the dose of the particular inhibitor used, A β (40) production was not always decreased (Klafki *et al.*, 1996; Citron *et al.*, 1996). Other experiments using lower doses of MG132 and calpeptin detected increases in both A β (40) and A β (42) levels (Yamazaki *et al.*, 1997). Nonetheless, the overall effect of these compounds was to differentially increase the ratio of A β (42)/A β (40) secretion, therefore arguing for the existence of distinct γ -secretases responsible for generating A β (40) or A β (42). Given that it is likely that these γ -secretase inhibitors act late in the secretory pathway (Higaki *et al.*, 1995), these results are in keeping with the idea that A β (42) would be less inhibited by these compounds than A β (40) because A β (42) is not the predominant A β species in the secreted A β pool (Turner *et al.*, 1996; Tienari *et al.*, 1997). Such evidence notwithstanding, it is still possible that a single γ -secretase might cleave APP at different sites depending upon the specific intracellular compartment where the cleavage occurs.

Whether or not there is one or multiple γ -secretases, a particularly puzzling aspect of γ -secretase function is how it cleaves a region of APP that is apparently buried in the membrane bilayer. Currently, there are no answers to this question. However, with the recent discovery that γ -secretase is active in the ER, it is intriguing to speculate that the specific properties of the ER membrane could play a role in the activity of γ -secretase. The lipid membrane bilayer of the ER possesses lower proportions of sphingolipids and cholesterol compared to membranes that compose more distal compartments of the secretory pathway, thus making the ER membrane comparatively thinner and more fluid (Orci *et al.*, 1981; Bretscher and Munro, 1993). Accordingly, it is plausible that while APP is located in the ER the A β (42), but not the A β (40), cleavage site might be accessible to a γ -secretase on the cytoplasmic side

of the ER. As APP trafficks into other domains of the cell where the bilayer becomes thicker the A β (42) cleavage site might, as has long been proposed, become embedded in the membrane (Figure 1). Under such circumstances other mechanisms must be proposed for how γ -secretase cleaves APP. Although the mechanisms underlying this process are currently unknown, this is not a biological conundrum restricted to APP metabolism. Misfolded integral membrane proteins, for example, must be dislocated from the ER membrane in order to undergo proteolytic digestion (Kopito, 1997). How this occurs is not well understood. A particularly fascinating example of such a process is mediated by two cytomegalovirus (CMV) proteins US2 or US11 (Weirtz *et al.*, 1996a,b). Through the activity of these viral proteins CMV evades the host immune system by selectively dislocating class 1 heavy chain molecules from ER membranes, after which they are rapidly degraded. How this process compares with endogenous cellular mechanisms for retrograde translocation of proteins from the ER is not known. However, it is reasonable to speculate that related mechanisms may play a role in normal protein metabolism and that γ -secretase activity may rely on similar cellular molecules to partially dislocate APP from membranes in order to generate A β .

The fact that both β - and γ -secretase are active in the ER/IC raises the question as to what known endoproteolytic activities are associated with the ER. A major function of the ER is to regulate the synthesis and assembly of proteins into conformationally specific molecules (Hurtley and Helenius, 1989). Proteins failing to assemble properly cannot leave the ER and must be disposed of (Rose and Doms, 1988). Thus a significant amount of cellular proteolysis is associated with the ER. Currently it is thought that much of this activity is accounted for by the action of the ubiquitin/proteasome protein degradation system (Goldberg and Rock, 1992; Rock *et al.*, 1994). The proteasome is a large multisubunit complex that is responsible for degrading ubiquitin conjugated proteins in an ATP-dependent manner (Hershko and Ciechanover, 1992; Rechsteiner *et al.*, 1993). Studies exploring the potential role of the proteasome in A β metabolism have yielded differing views. One report suggested that the proteasome may mediate γ -secretase activity because a reporter peptide similar to the APP γ -secretase cleavage site appeared to be cleaved by the proteasome (Mundy, 1994). However, other investigators have argued that A β inhibits the proteasome (Gregori *et al.*, 1995; Gregori *et al.*, 1997). Well-characterized compounds that block the proteasome, including lactacystin which is considered the most specific inhibitor of the proteasome (Dick *et al.*, 1997), actually increased the production of both A β (40) and A β (42) (Yamazaki *et al.*, 1997). Thus, at this time it is not clear whether the proteasome could have γ -secretase activity.

BIOLOGICAL SIGNIFICANCE OF THE ER/IC PATHWAY

In addressing the potential biological significance of the ER/IC pathway in AD it is important to note that, compared to overall A β production, the ER/IC pathway generating A β (42) appears to produce relatively little A β . Nevertheless, it is possible that the ER/IC pathway may still be highly significant in the AD disease process because of the critical role A β (42) is thought to play in the pathogenesis of this disease. In addition, the presenilins are localized in the ER/IC (Kovacs *et al.*, 1996; Cook *et al.*, 1996; Lah *et al.*, 1997) and mutations in PS1 and PS2 associated with FAD cause increased levels of A β (42) production (Scheuner *et al.*, 1996; Duff *et al.*, 1996; Lemere *et al.*, 1996; Tomita *et al.*, 1997). These data raise the possibility that the activities of three distinct genes associated with Alzheimer's

disease (APP, PS1, and PS2) may interact in a common pathway involving pathogenic alterations in APP processing that may occur in the ER/IC. In keeping with this possibility recent work indicates that newly synthesized immature APP molecules, rather than mature processed forms of APP, specifically interact with PS1 and PS2 in the ER (Weidemann *et al.*, 1997; Xia *et al.*, 1997). Although, mutations in the presenilins have not been shown to alter interactions between APP and PS1 or PS2, an attractive hypothesis is that normal trafficking of APP through the ER/IC is modulated by chaperone-like interactions between APP and the presenilins. Mutations in PS1 or PS2 may alter such interactions to favor increased production of A β (42).

Another important issue pertaining to the potential biological significance of the ER/ IC processing pathway is whether A β (42) made in the ER/IC is secreted. Our work indicates that this intracellular pool of A β (42) is either not secreted or only secreted inefficiently (Cook *et al.*, 1997). As a result, the ER/IC pathway does not make a significant contribution to A β secretion, which is thought to be a critical step in the deposition of A β into plaques. However, the studies discussed above indicating that production of intracellular A β is largely a property of neurons and that highly amyloidogenic A β (42) is produced by the ER/IC pathway calls for a reexamination of this issue. It is plausible, for example, that intracellular A β (42) from dead or dying neurons could form a nidus for the subsequent deposition of A β derived from larger extracellular sources. Support for this notion comes from observations that A β immuno-reactivity can be seen inside cells associated with neurofibrillary tangles (Grundke, *et al.*, 1989; Perry *et al.*, 1992; Murphy *et al.*, 1994). Furthermore, Martin *et al.*, (1994) have reported that non-fibrillar A β can be found inside neurons and non-neuronal cells prior to the appearance of extracellular A β deposits in macaque brains. Similarly, recent experiments reported that TUNEL-positive neurons in AD brains may express intracellular A β (LaFerla *et al.*, 1997). Cell death is a prominent feature of AD (Cotman and Su, 1996; Gomez-Isla *et al.*, 1996), and owing to the well-established toxicity of A β (Inversen *et al.*, 1995) accumulation of intracellular A β , such as that derived from the ER/IC, could contribute to the destruction of neurons in AD. Consistent with this notion, a recently discovered intracellular protein found in the ER, termed ERAB, binds A β and may play a role in A β -mediated neurotoxicity (Yan *et al.*, 1997).

It is interesting to note that accumulation of abnormally processed proteins may play a role in several diseases including: AD, characterized by intracellular inclusions of tau protein in the form of neurofibrillary tangles (Goedert *et al.*, 1991; Lee and Trojanowski, 1992); spinocerebellar ataxia type 3, characterized by intranuclear inclusions of ataxin-3 (Paulson *et al.*, 1997); Huntington's disease, characterized by intranuclear inclusion of huntingtin (DiFiglia *et al.*, 1997; Davies *et al.*, 1997; Scherzinger *et al.*, 1997), and Parkinson's disease and Lewy Body disease, characterized by intracellular Lewy bodies composed at least in part by α -synuclein (Polymeropoulos *et al.*, 1997; Spillantini *et al.*, 1997). Further investigations into the mechanisms underlying the production and metabolism of intracellular A β may offer new perspectives regarding the molecular underpinnings of AD and lead to additional insights into the mechanistic features AD may share in common with a broad class of neurodegenerative disorders.

REFERENCES

- Alzheimer's Disease Collaborative Group (1995) The structure of the presenilin-1 (S182) gene and identification of 6 novel mutations in early-onset AD families. *Nature Genetics*, **11**, 219–222.
- Arai, H., Lee, V.M.-Y., Messinger, M.L., Greenberg, B.D., Lowery, D.E., and Trojanowski, J.Q. (1991) Expression patterns of β -amyloid precursor protein (β -APP) in neural and nonneural human tissues from Alzheimer's disease and control subjects. *Annals of Neurology*, **30**, 686–693.
- Bretscher, M.S., and Munro, S. (1993) Cholesterol and the Golgi apparatus. *Science*, **261**, 1280–1281.
- Busciglio, J., Gabuzda, D., Matsudaira, P., and Yankner, B. (1993) Generation of beta-amyloid in the secretory pathway in neuronal and nonneuronal cells. *Proceedings of the National Academy of Science*, **90**, 2092–2096.
- Cai, X.-D., Golde, T.E., and Yankner, S.G. (1993) Release of excess amyloid beta protein from a mutant amyloid beta protein precursor. *Science*, **259**, 514–516.
- Chartier-Harlin, M.-C., Crawford, F., Houlden, H., Warren, A., Hughes, D., Fidani, L., Goate, A., Rossor, M., Roques, P., Hardy, J., and Mullan, M. (1991) Early-onset Alzheimer's disease caused by mutations at codon 717 of the β -amyloid precursor protein gene. *Nature*, **353**, 844–846.
- Checler, F. (1995) Processing of the β -amyloid precursor protein and its regulation in Alzheimer's disease. *Journal of Neurochemistry*, **65**, 1431–1444.
- Chyung, A.S.C., Greenberg, B.D., Cook, D.G., Doms, R.W., and Lee, V.M.-Y. (1997) Novel β -secretase cleavage of β -amyloid precursor protein in the endoplasmic reticulum/intermediate compartment of NT2N cells. *Journal of Cell Biology*, **138**, 671–680.
- Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A. Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., and Selkoe, D.J. (1992) Mutation of the β -amyloid precursor protein in familial Alzheimer's disease increases β -protein production. *Nature*, **360**, 672–674.
- Citron, M., Vigo-Pelfrey, C., Teplow, D.B., Miller, C., Schenk, D., Johnston, J., Winblad, B., Venzelos, N., Lannfelt, L., and Selkoe, D.J. (1994) Excessive production of amyloid β -protein by peripheral cells of symptomatic and presymptomatic patients carrying the Swedish familial Alzheimer disease mutation. *Proceedings of The National Academy of Science*, **91**, 11993–11997.
- Citron, M., Teplow, D.B., and Selkoe, D.J. (1995) Generation of amyloid β protein from its precursor is sequence specific. *Neuron*, **14**, 661–670.
- Citron, M., Diehl, T.S., Gordon, G., Biere, A.L., Seubert, P., and Selkoe, D.J. (1996) Evidence that the 42- and 40-amino acid forms of amyloid protein are generated from the amyloid precursor protein by different protease activities. *Proceedings of the National Academy of Science*, **93**, 13170–13175.
- Cook, D.G., Sung, J.C., Golde, T.E., Felsenstein, K.M., Wojczyk, B.S., Tanzi, R.E., Trojanowski, J.Q., Lee, V.M.-Y., and Doms, R.W. (1996) Expression and analysis of presenilin 1 in a human neuronal system: localization in cell bodies and dendrites. *Proceedings of the National Academy of Science*, **93**, 9223–9228.
- Cook, D.G., Forman, M.S., Sung, J.C., Leight, S., Kolson, D.L., Iwatsubo, T., Lee, V.M.-Y., and Doms, R.W. (1997) Alzheimer A β (42) is generated in the endoplasmic reticulum/intermediate compartment of NT2N cells. *Nature Medicine*, **3**, 1021–1023.
- Cotman, C.W., and Su, J.H. (1996) Mechanisms of neuronal death in Alzheimer's disease. *Brain Pathology*, **6**, 493–506.

- Davies, S.W., Turmain, M., Cozens, B.A., DiFiglia, M., Sharp, A.H., Ross, C.A., Scherzinger, E., Wanker, E.E., Mangiarini, L., and Bates, G.P. (1997) Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. *Cell*, **90**, 537–548.
- Davis, R.E., Miller, S., Herrnstadt, C., Ghosh, S.S., Fahy, E., Shinobu, L.A., Galasko, D., Thal, L.J., Beal, M.F., Howell, N., and Parker, W.D. (1997) Mutations in mitochondrial cytochrome c oxidase genes segregate with late-onset Alzheimer's disease. *Proceedings of the National Academy of Science*, **94**, 4526–4531.
- DeStrooper, B., Umans, L., VanLeuven, F., and VanDenBerghe, H. (1993) Study of the synthesis and secretion of normal and artificial mutants of murine amyloid precursor protein (APP): cleavage of APP occurs in a late compartment of the default secretory pathway. *Journal of Cell Biology*, **121**, 295–304.
- DeStrooper, B., Simons, M., Multhaup, G., Vanleuven, F., Beyreuther, K., and Dotti, C.G. (1995) Production of intracellular amyloid-containing fragments in hippocampal-neurons expressing human amyloid precursor protein and protection against amyloidogenesis by subtle amino-acid substitutions in the rodent sequenc. *EMBO Journal*, **14**, 4932–4938.
- Dick, L.R., Cruikshank, A.A., Destree, A.T., Grenier, L., McCormack, T.A., Melandri, F.D., Nunes, S.L., Palombella, V.J., Parent, L.A., Plamondon, L., and Stein, R.L. (1997) Mechanistic studies on the inactivation of the proteasome by lactacystin in cultured cells. *Journal of Biological Chemistry*, **272**, 182–188.
- 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.
- Doms, R.W., Russ, G., and Yewdell, J.W. (1989) Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. *Journal of Cell Biology*, **109**, 61–72.
- Duff, K., Eckman, C., Zehr, C., Yu, X., Prada, C.-M., Perez-tur, J., Mutton, M., Buee, L., Harigaya, Y., Yager, D., Morgan, D., Gordon, M.N., Holcomb, L., Refolo, L., Zenk, B., Hardy, J., and Younkin, S. (1996) Increased amyloid- β 42(43) in brains of mice expressing mutant presenilin 1. *Nature*, **383**, 710–713.
- Esch, F.S., Keim, P.S., Beattie, E.C., Blacher, R.W., Culwell, A.R., Oltersdorf, T., McClure, D., and Ward, P.J. (1990) Cleavage of amyloid β peptide during constitutive procesing of its precursor. *Science*, **248**, 1122–1124.
- Felsenstein, K.M., Hunihan, L.W., and Roberts, S.B. (1994) Altered cleavage and secretion of a recombinant β -APP bearing the Swedish familial Alzheimer's disease mutation. *Nature Genetics*, **6**, 251–256.
- Forman, M.S., Cook, D.G., Leight, S., Doms, R.W., and Lee, V.M.-Y (1997) Differential effects of the Swedish mutant amyloid precursor protein on β -amyloid accumulation and secretion in neurons and non-neuronal cells. *Journal of Biological Chemistry*, **51**, 32247–32253.
- Fukumoto, H., AsamiOdaka, A., Suzuki, N., Shimada, H., Ihara, Y., and Iwatsubo, T. (1996) Amyloid- β protein deposition in normal aging has the same characteristics as that in Alzheimer's-disease-predominance of A β 42(43) and association of A- β -40 with cored plaques. *American Journal of Pathology*, **148**, 259–265.
- Fuller, S.J., Storey, E., Li, Q.X., Smith, A.I., Beyreuther, K., and Masters, C.L. (1995) Intracellular production of beta A4 amyloid of Alzheimer's disease: modulation by phosphoramidon and lack of coupling to the secretion of the amyloid precursor protein. *Biochemistry*, **34**, 8091–8098.
- Furukawa, K., Barger, S.W., Blalock, E.M., and Mattson, M.P. (1996) Activation of K⁺ channels and suppression of neuronal activity by secreted β -amyloid-precursor protein. *Nature*, **379**, 74–78.
- Gabuzda, D., Busciglio, J., Chen, L.B., Masudaira, P., and Yankner, B.A. (1994) Inhibition of energy metabolism alters the processing of amyloid precursor protein and induces a potentially amyloidogenic derivative. *Journal of Biological Chemistry*, **269**, 13623–13628.

- Games, D., Adams, D., Alessandrini, R., Barbour, R., Berthelette, P., Blackwell, C., Carr, T., Clemens, J., Donaldson, T., Gillespie, F., Guido, T., Hagoplan, 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., Sorlano, F., Tan, H., Vitale, J., Wadsworth, S., Wolozin, B., and Zhao, J. (1995) Alzheimer-type neuropathology in transgenic mice overexpressing V717F β -amyloid precursor protein. *Nature*, **373**, 523–527.
- Giaccone, G., Tagliavini, F., Linoli, G., Bouras, C., Frigerio, L., Frangione, B., and Bugiani, O. (1989) Down patients: extracellular preamyloid deposits precede neuritic degeneration and senile plaques. *Neuroscience Letters*, **97**, 232–238.
- Glennner, G.G., and Wong, C.W. (1984) Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein. *Biochemical and Biophysical Research Communications*, **122**, 1131–1135.
- Goate, A., Chartier-Harlin, M.-C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., Mant, R., Newton, P., Rooke, K., Roques, P., Talbot, C., Pericak-Vance, M., Roses, A., Williamson, R., Rossor, M., Owen, M., and Hardy, J. (1991) Segregation of a missense mutation in the amyloid precursor gene with familial Alzheimer's disease. *Nature*, **349**, 704–706.
- Goedert, M. (1987) Neuronal localization of amyloid β protein precursor mRNA in normal brain and Alzheimer's disease. *EMBO Journal*, **6**, 3627–3632.
- Goedert, M., Spillantini, M.G., and Crowther, R.A. (1991) Tau proteins and neurofibrillary degeneration. *Brain Pathology*, **1**, 279–86.
- Goldberg, A.L., and Rock, K.L. (1992) Proteolysis, proteasomes and antigen presentation. *Nature*, **357**, 375–379.
- Golde, T.E., Estus, S.G., Usiak, M., Younkin, L.H., and Younkin, S.G. (1990) Expression of β amyloid protein precursor mRNAs: Recognition of a novel alternatively spliced form and quantitation in Alzheimer's disease using PCR. *Neuron*, **4**, 253–267.
- Golde, T.E., Estus, S., Younkin, L.H., Selkoe, D.J., and Younkin, S.G. (1992) Processing of the amyloid protein precursor to potentially amyloidogenic derivatives. *Science*, **255**, 728–730.
- Golde, T.E., and Younkin, S.G. (1995) Processing of the amyloid β protein precursor. In *Neuroscience Perspectives: Neurofibrillary Tangles and Amyloid Precursor Protein in Alzheimer's disease* (eds A.Goate and F.Ashall), pp. 113–141: Academic Press.
- Goldgaber, D., Lerman, M.I., McBride, O.W., Saffiotti, U., and Gajdusek, D.C. (1987) Characterization and chromosomal localization of a cDNA encoding brain amyloid of Alzheimer's disease. *Science*, **235**, 877–880.
- Gomez-Isla, T., Price, J.L., McKeel, D.W., Morris, J.C., Growdon, J.H., and Hyman, B.T. (1996) Profound loss of layer II entorhinal cortex neurons occurs in very mild Alzheimer's disease. *Journal of Neuroscience*, **16**, 4491–4500.
- Gowing, E., Roher, A.E., Woods, A.S., Cotter, R.J., Chaney, M., Little, S.P., and Ball, M.J. (1994) Chemical characterization of A β 17–42 peptide, a component of diffuse amyloid deposits of Alzheimer's disease. *Journal of Biological Chemistry*, **269**, 10987–10990.
- Gregori, L., Fuchs, C., Figueiredo-Pereira, M.E., Nostrand, W.E.V., and Goldgaber, D. (1995) Amyloid beta-protein inhibits ubiquitin-dependent protein degradation *in vitro*. *Journal of Biological Chemistry*, **270**, 19702–19708.
- Gregori, L., Hainfeld, J.F., Simon, M.N., and Goldgaber, D. (1997) Binding of amyloid β protein to the 20 S proteasome. *Journal of Biological Chemistry*, **272**, 58–62.
- Grundke-Iqbal, I., Iqbal, K., George, L., Tung, Y.C., Kim, K.S., and Wisniewski, H.M. (1989) Amyloid protein and neurofibrillary tangles coexist in the same neuron in Alzheimer disease. *Proceedings of the National Academy of Science*, **86**, 2853–2857.

- Hershko, A., and Ciechanover A. (1992) The ubiquitin system for protein degradation. *Annual Review of Biochemistry*, **61**, 761–807.
- Haass, C., Koo, E.H., Mello, A., Hung, A.Y., and Selkoe, D.J. (1992a) Targeting of cell-surface β -amyloid precursor protein to lysosomes: alternative processing into amyloid-bearing fragments. *Nature*, **357**, 500–503.
- Haass, C., Schlossmacher, M.G., Hung, A.Y., Bibo-Pfeiffer, C., Mellon, A., Ostaszewski, B.L., Lieberburg, I., Koo, E.H., Schenk, D., Teplow, D.B., and Selkoe, D.J. (1992b) Amyloid β -peptide is produced by cultured cells during normal metabolism. *Nature*, **359**, 322–325.
- Haass, C., Hung, A.V., Schlossmacher, M.G., Teplow, D.B., and Selkoe, D.J. (1993) Beta-Amyloid peptide and a 3-kDa fragment are derived by distinct cellular mechanisms. *Journal of Biological Chemistry*, **268**, 3021–3024.
- Haass, C., Koo, E.H., Teplow, D.B., and Selkoe, D.J. (1994) Polarized secretion of β -amyloid precursor protein and amyloid β -peptide in MDCK cells. *Proceedings of the National Academy of Science*, **91**, 1564–1568.
- Haass, C., Lemere, C.A., Capell, A., Citron, M., Seubert, P., Schenk, D., Lannfelt, L., and Selkoe, D.J. (1995) The Swedish mutation causes early-onset Alzheimer's disease by β -secretase cleavage within the secretory pathway. *Nature Medicine*, **1**, 1291–1296.
- 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 β 40/42 amyloid peptides. *Nature Medicine*, **3**, 1016–1017.
- Higaki, J., Quon, D., Zhong, Z., and Cordell, B. (1995) Inhibition of β -amyloid formation identifies proteolytic precursors and subcellular site of catabolism. *Neuron*, **14**, 651–659.
- 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.
- Hung, A.Y., Koo, E.H., Haass, C., and Selkoe, D.J. (1992) Increased expression of β -amyloid precursor protein during neuronal differentiation is not accompanied by secretory cleavage. *Proceedings of the National Academy of Science*, **89**, 9439–9443.
- Hurtley, S.M., and Helenius, A. (1989) Protein oligomerization in the endoplasmic reticulum. *Annual Review of Cell Biology*, **5**, 277–307.
- Iverson, L.L., Mortishire-Smith, R.J., Pollack, S.J., and Shearman, M.S. (1995) The toxicity *in vitro* of β -amyloid protein. *Biochemical Journal*, **311**, 1–16.
- Iwatsubo, T., Odaka, A., Suzuki, N., Mizusawa, H., Nukina, N., and Ihara, Y. (1994) Visualization of A β 42(43) and A β 40 in senile plaques with specific A β monoclonals: evidence that an initially deposited species is A β 42(43). *Neuron*, **13**, 45–53.
- Iwatsubo, T., Mann, D.M.A., Odaka, A., Suzuki, N., and Ihara, Y. (1995) Amyloid-beta protein (A β) deposition A β 42(43) precedes A β 40 in Down syndrome. *Annals of Neurology*, **37**, 294–299.
- Jarrett, J.T., Berger, E.P., and Lansbury, P.T. (1993) The carboxy terminus of β amyloid protein is critical for the seeding of amyloid formation: Implications for pathogenesis of Alzheimer's disease. *Biochemistry*, **32**, 4693–4697.
- Jarrett, J.T., and Lansbury, P.T. (1993) Seeding “one dimensional crystallization” of amyloid: A pathogenic mechanism in Alzheimer's disease and Scrapie? *Cell*, **73**, 1055–1058.
- Johnson-Wood, K., Lee, M., Motter, R., Hu, K., Gordon, G., Barbour, R., Khan, K., Gordon, M., Tan, H., Games, D., Lieberburg, I., Schenk, D., Seubert, P., and McConlogue, L. (1997) Amyloid precursor protein processing and A β 42 deposition in a transgenic mouse model of Alzheimer disease. *Proceedings of the National Academy of Science*, **94**, 1550Z–1555.

- Kang, J., Lemaire, H.-G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grzeschik, K.-H., Multhaup, G., Beyreuther, K., and Muller-Hill, B. (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature*, **325**, 733–736.
- Kang, J., and Muller-Hill, B. (1990) Differential splicing of Alzheimer's disease amyloid A4 precursor RNA in rat tissues: PreA4–695 mRNA is predominantly produced in rat and human brain. *Biochemical and Biophysical Research Communications*, **166**, 1192–1200.
- Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S., and Ito, H. (1988) Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity. *Nature*, **331**, 530–532.
- Klafki, H.W., Abramowski, D., Swoboda, R., Paganetti, P.A., and Staufenbiel, M. (1996) The carboxyl termini of β amyloid peptides 1–40 and 1–42 are generated by distinct β -secretase activities. *Journal of Biological Chemistry*, **271**, 28655–28659.
- Knops, J., Gandy, S., Greenwood, P., Liebersburg, I., and Sinhas, S. (1993) Serine phosphorylation of the secreted extracellular domain of APP. *Biochemical and Biophysical Research Communications*, **197**, 380–385.
- Koo, E.H., Sisodia, S.S., Cork, L.C., Unterbeck, A., Bayney, R.M., and Price, D.L. (1990) Differential expression of amyloid precursor protein mRNAs in cases of Alzheimer's disease and in aged nonhuman primates. *Neuron*, **2**, 97–104.
- Koo, E.H., and Squazzo, S. (1994) Evidence that production and release of amyloid beta-protein involves the endocytic pathway. *Journal of Biological Chemistry*, **269**, 17386–17389.
- Kopito, R.R. (1997) ER quality control: the cytoplasmic connection. *Cell*, **88**, 427–430.
- Kovacs, D.M., Fausett, H.J., Page, K.J., Kim, T.-W., Moir, R.D., Merriam, D.E., Hollister, R.D., Hallmark, O.G., Mancini, R., Felsentein, K.M., Hyman, B.T., Tanzi, R.E., and Wasco, W. (1996) Alzheimer-associated presenilins 1 and 2: Neuronal expression in brain and localization to intracellular membranes in mammalian cells. *Nature Medicine*, **2**, 224–229.
- Kuentzel, S.L., Ali, S.M., Greenberg, B.D., and Raub, T.J. (1993) The Alzheimer β -amyloid protein precursor/protease nexin II is cleaved by secretase in a trans-Golgi secretory compartment in human neuro-glioma cells. *Biochemical Journal*, **295**, 367–378.
- 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. *Journal of Clinical Investigation*, **100**, 310–320.
- Lah, J., Heilman, C., Nash, N., Rees, H., Yi, H., Counts, S., and Levey, A. (1997) Light and Electron Microscopic Localization of Presenilin-1 in Primate Brain. *Journal of Neuroscience*, **17**, 1971–1980.
- Lai, A., Sisodia, S., and Trowbridge, I.S. (1995) Characterization of sorting signals in the β -amyloid precursor protein cytoplasmic domain. *J. Biol Chem*, **270**, 3565–3573.
- Lee, V.M.-Y., and Trojanowski, J.Q. (1992) The disordered neuronal cytoskeleton in Alzheimer's disease. *Current Opinion in Neurobiology*, **2**, 653–656.
- Lemere, C.A., Lopera, F., Kosik, K.S., Lendon, C.L., Ossa, J., Saido, T.C., Yamaguchi, H., Ruiz, A., Martinez, A., Madrigal, L., Hincapie, L., Arango-L, J.C., Anthony, D.C., Koo, E.H., Goate, A.M., Selkoe, D.J., and Arango-V, J.C. (1996) The e280a presenilin 1 alzheimer mutation produces increased a β 42 deposition and severe cerebellar pathology. *Nature Medicine*, **2**, 1146–1150.
- Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D.M., Oshima, J., Pettingell, W.H., Yu, C., Jondro, P.D., Schmidt, S.D., Wang, K., Crowley, A.C., Fu, Y.-H., Guenette, S.Y., Galas, D., Nemens, E., Wijsman, E.M., Bird, T.D.,

- Schellenberg, G.D., and Tanzi, R.E. (1995a) Candidate gene for the Chromosome 1 Familial Alzheimer's Disease Locus. *Science*, **269**, 973–977.
- Levy-Lahad, E., Wijsman, E.M., Nemens, E., Anderson, L., Goddard, K.A.B., Weber, J.L., Bird, T.D., and Schellenberg, G.D. (1995b) A familial Alzheimer's disease locus on chromosome 1. *Science*, **269**, 970–973.
- Liljestrom, P., and Garoff, H. (1991) A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. *Bio/Technology*, **9**, 1356–1361.
- Lippincott-Schwartz, J., Yuan, L.C., Bonifacino, J.S., and Klausner, R.D. (1989) Rapid redistribution of Golgi proteins into the endoplasmic reticulum in cells treated with Brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell*, **56**, 801–813.
- Luo, L., Tully, T., and White, K. (1992) Human amyloid precursor protein ameliorates behavioral deficit of flies. *Neuron*, **9**, 595–605.
- Mann, D.M.A., Jones, D., Prinja, D., and Purkiss, M.S. (1990) The prevalence of amyloid (A4) protein deposits within the cerebral and cerebellar cortex in Down's syndrome and Alzheimer's disease. *Acta Neuropathology*, **80**, 318–327.
- Marquez-Sterling, N.R., Lo, A.C.Y., Sisodia, S.S., and Koo, E.H. (1997) Trafficking of cell-surface β -amyloid precursor protein: evidence that a sorting intermediate participates in synaptic vesicle recycling. *Journal of Neuroscience*, **17**, 140–151.
- Martin, L.J., Pardo, C.A., Cork, L.C., and Price, D.L. (1994) Synaptic pathology and glial responses to neuronal injury precede the formation of senile plaques and amyloid deposits in the aging cerebral cortex. *American Journal of Pathology*, **145**, 1358–1381.
- Martin, B.L., Schrader-Fischer, G., Busciglio, J., Duke, M., Paganetti, P., and Yankner, B.A. (1995) Intracellular accumulation of β -amyloid in cells expressing the Swedish mutant amyloid precursor protein. *Journal of Biological Chemistry*, **270**, 26727–26730.
- 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. *Proceedings of the National Academy of Science*, **82**, 4245–4249.
- Mullan, M., Crawford, F., Axelman, K., Houlden, H., Lilius, L., Winblad, B., and Lannfelt, L. (1992) A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of β -amyloid. *Nature genetics*, **1**, 345–347.
- Muller, U., Cristina, N., Li, Z.W., Wolfer, D.P., Lipp, H.P., Rulicke, T., Brandner, S., Aguzzi, A., and Weissmann, C. (1994) Behavioral and anatomical deficits in mice homozygous for a modified β -amyloid precursor protein. *Cell*, **79**, 755–765.
- Mundy, D.I. (1994) Identification of the multicatalytic enzyme as a possible gamma-secretase for the amyloid precursor protein. *Biochemical and Biophysical Research Communications*, **204**, 333–341.
- Murphy, G.M., Forno, L.S., Higgins, L., Scardina, J.M., Eng, L.F., and Cordell, B. (1994) Development of a monoclonal antibody specific for the COOH-terminal of β -amyloid 1–42 and its immunohistochemical reactivity in Alzheimer's disease and related disorders. *American Journal of Pathology*, **144**, 1082–1088.
- Murrell, J., Farlow, M., Ghetti, B., and Benson, M.D. (1991) A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. *Science*, **254**, 97–99.
- Neve, R.L., Finch, E.A., and Dawes, L.R. (1988) Expression of the Alzheimer amyloid precursor gene transcripts in the human brain. *Neuron*, **1**, 669–677.

- Nordstedt, C., Caporaso, G.L., Thyberg, J., Gandy, S.E., and Greengard, P. (1993) Identification of the Alzheimer beta/A4 amyloid precursor protein in clathrin-coated vesicles purified from PC 12 cells. *Journal of Biological Chemistry*, **268**, 608–612.
- Oltersdorf, T., Ward, P.J., Henriksson, T., Beattie, E.C., Neve, R., Lieberburg, I., and Fritz, L.C. (1990) The Alzheimer amyloid precursor protein: identification of a stable intermediate in the biosynthetic/degradative pathway. *Journal of Biological Chemistry*, **265**, 4492–4497.
- Orci, L., Montesano, R., Meda, P., Malaisse-Lagae, F., Brown, D., Perrelet, A., and Vassalli, P. (1981) Heterogeneous distribution of filipin-cholesterol complexes across the cisternae of the Golgi apparatus. *Proceedings of the National Academy*, **78**, 293–297.
- Palmer, A.M. (1996) Neurochemical studies of Alzheimer's disease. *Neurodegeneration*, **5**, 381–391.
- Paulson, H.L., Perez, M.K., Trotter, Y., Trojanowski, J.Q., S.H.Subramony, Das, S.S., Vig, P., Mandel, J.L., Fischbeck, K.H., and Pittman, R.N. (1997) Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3. *Neuron*, **19**, 333–344.
- Peraus, G.C, Masters, C.L., and Beyreuther (1997) Late compartments of amyloid precursor protein transport in SY5Y cells are involved in β -amyloid secretion. *Journal of Neuroscience*, **17**, 7714–7724
- Perez, R.G., Squazzo, S.L., and Koo, E.H. (1996) Enhanced release of A β from codon 670/671 "Swedish" mutant β amyloid precursor protein occurs in both secretory and endocytic compartments. *Journal of Biological Chemistry*, **271**, 9100–9107.
- Perry, G., Cras, P., Siedlak, S.L., Tabaton, M, and Kawai, M. (1992) β -protein immunoreactivity is found in the majority of neurofibrillary tangles of Alzheimer's disease. *American Journal of Pathology*, **140**, 283–290.
- 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., Johnson, W.G., Lazzarini, A.M., Duvoisin, R.C., DiLorio, G., Golbe, L.I., and Nussbaum, R.L. (1997) Mutation in the α -synuclein gene identified in families with Parkinson's disease. *Science*, **276**, 2045–2047.
- Ponte, P., Gonzalez-DeWhitt, P., Schilling, J., Miller, J., Hsu, D., Greenberg, B., Davis, K., Wallace, W., Lieberburg, I., Fuller, F., and Cordell, B. (1988) A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors. *Nature*, **331**, 525–527.
- Robakis, N.K., Ramakrishna, N., Wolfe, G., and Wisniewski, H.M. (1987) Molecular cloning and characterization of a cDNA encoding the cerebrovascular and the neuritic plaque amyloid peptides. *Proceedings of the National Academy of Science*, **84**, 4190–4194.
- 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.
- Rechsteiner, M., Hoffman, L., and Dubiel, W. (1993) The multicatalytic and 26S proteases. *Journal of Biological Chemistry*, **268**, 6065–6068.
- Rogaev, E.I., Sherrington, R., Levesque, G., Ikeda, M., and St. George Hyslop, P.H. (1995) Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. *Nature*, **376**, 775–778.
- Rose, J.K., and Doms, R.W. (1988) Regulation of protein export from the endoplasmic reticulum. *Annual Review of Cell Biology*, **4**, 257–288.
- Roses, A.D. (1995) Perspective: on the metabolism of apolipoprotein E and Alzheimer's diseases. *Experimental Neurology*, **132**, 149–156.

- Sambamurti, K., Shioi, J., Anderson, J.P., Pappolla, M.A., and Robakis, N.K. (1992) Evidence for intracellular cleavage of the Alzheimer's amyloid precursor. *Journal of Neuroscience Research*, **33**, 319–329.
- Scherzinger, E., Lurz, R., Turmaine, M., Mangiarini, L., Holenbach, 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.
- Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T.D., Hardy, J., Mutton, M., Kukull, W., Larson, E., Levy-Lahad, E., Viitanen, M., Peskind, E., Poorkaj, P., G.Schellenberg, Tanzi, R., Wasco, W., Lannfelt, L., Selkoe, D., and Younkin, S. (1996) Secreted amyloid β -protein similar to that in the senile plaques of Alzheimer's disease is increased *in vivo* by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nature Medicine*, **2**, 864–870.
- Selkoe, D.J. (1994) Normal and abnormal biology of the β -amyloid precursor protein. *Annual Review of Neuroscience*, **17**, 489–517.
- Selkoe, D.J. (1997a) Alzheimer's disease: genotypes, phenotypes and treatments. *Science*, **275**, 630–631.
- Selkoe, D.J. (1997b) Cellular and molecular biology of beta-amyloid precursor and Alzheimer's Disease. In *The molecular and genetic basis of neurological disease*, (eds. S.B.Prusiner, R.N. Rosenberg, S.D.Mauro, *et al.*), pp. 601–612. Boston, MA: Butterworth Heineman Press.
- Seubert, P., Oltersdorf, T., Lee, M.G., Barbour, R., Blomquist, C., Davis, D.L., Bryant, K., Fritz, L.C., Galasko, D., Thal, L.J., Lieberburg, I., and Schenk, D.B. (1993) Secretion of β -amyloid precursor protein cleaved at the amino terminus of the β -amyloid peptide. *Nature*, **361**, 260–263.
- Sherrington, R., Rogaev E.I., Liang, Y., Rogaeva, E.I., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., Tsuda, T., Mar, L., Foncin, J.-K., Bruni, A.C., Montesi, M.P., Sorbi, S., Rainero, I., Pinessi, L., Nee, L., Chumakov, I., Pollen, D., Brookes, A., Sanseau, P., Polinsky, R.J., Wasco, W., Da Silva, H.A.R., Haines, J.L., Percek-Vance, M.A., Tanzi, R.E., Roses, A.D., Fraser, P.E., Romens, J.M., and St George-Hyslop, P.H. (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature*, **375**, 754–760.
- Shoji, M., Golde, T.E., Ghiso, J., Cheung, T.T., Estus, S., Shaffer, L.M., Cai, X., McKay, D.M., Tintner, R., Frangione, B., and Younkin, S.G. (1992) Production of the Alzheimer amyloid β protein by normal proteolytic processing. *Science*, **258**, 126–129.
- Simons, M., Strooper, B.D., Multhaup, G., Tienari, P.J., Dotti, C.G., and Beyreuther, K. (1996) Amyloidogenic processing of the human amyloid precursor protein in primary cultures of rat hippocampal neurons. *Journal of Neuroscience*, **16**, 899–908.
- Sisodia, S.S., Koo, E.H., Beyreuther, K., Unterbeck, A., and Price, D.L. (1990) Evidence that β -amyloid protein in Alzheimer's disease is not derived by normal processing. *Science*, **248**, 492–495.
- Sisodia, S.S. (1992) β -amyloid precursor protein cleavage by a novel membrane bound protease. *Proceedings of the National Academy of Science*, **89**, 6075–6079.
- Small, D.H., Nurcombe, V., Reed, G., Clariss, H., Moir, R., Beyreuther, K., and Masters, C.L. (1994) A heparin-binding domain in the amyloid protein precursor of Alzheimer's disease is involved in the regulation of neurite outgrowth. *Journal of Neuroscience*, **14**, 2117–2127.
- Spillantini, M.G., Schmidt, M.L., Lee, V.M.-Y., Trojanowski, J.Q., Jakes, R., and Goedert, M. (1997) α -synuclein in Lewy bodies. *Nature*, **388**, 839–840.
- Suzuki, N., Cheung, T.T., Cai, X.-D., Odaka, A., Otvos, L., Eckman, C., Golde, T.E., and Younkin, S.G. (1994) An increased percentage of long amyloid β protein is secreted by familial amyloid β protein precursor (β APP 717) mutants. *Science*, **264**, 1336–1340.

- Tagliavini, F., Giaccone, G., Frangione, B., and Bugiani, O. (1988) Preamyloid deposits in the cerebral cortex of patients with Alzheimer's disease and nondemented individuals. *Neuroscience Letters*, **93**, 191–196.
- Tanzi, R.E., Gusella, J.F., Watkins, P.C., Bruns, G.A.R., 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.
- Tanzi, R.E., McClatchey, A.I., Lamperti, E.D., Villa-Komaroff, L., Gusella, J.F., and Neve, R.L. (1988) Protease inhibitor domain encoded by an amyloid protein precursor mRNA associated with Alzheimer's disease. *Nature*, **331**, 528–530.
- Thinakaran, G., Teplow, D.B., Siman, R., Greenberg, B., and Sisodia, S.S. (1996) Metabolism of the "Swedish" amyloid precursor protein variant in neuro2a (N2a) cells. Evidence that cleavage at the "beta secretase" site occurs in the golgi apparatus. *Journal of Biological Chemistry*, **271**, 9390–9397.
- 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 β -amyloid species are generated by distinct mechanisms in cultured hippocampal neurons. *Proceedings of the National Academy of Science*, **94**, 4125–4130.
- Tomita, T., Maruyama, K., T.Saido, Kume, H., Shinozaki, K., Tokuhiro, S., Capell, A., Walter, J., Grünberg, J., Haass, C., Iwatsubo, T., and Obata, K. (1997) The presenilin 2 mutation (N141I) linked to familial Alzheimer disease (Volga German families) increases the secretion of amyloid protein ending at the 42nd (or 43rd) residue. *Proceedings of the National Academy of Science*, **94**, 2025–2030.
- Turner, R.S., Suzuki, N., Chyung, A.S.C., Younkin, S.G., and Lee, V.M.-Y. (1996) Amyloid β 40 and β 42 are generated intracellularly in human neurons and their secretion increases with maturation. *Journal of Biological Chemistry*, **271**, 8966–8970.
- Weidemann, A., König, G., Bunke, D., Fischer, P., Salbaum, J.M., Masters, C.L., and Beyreuther, K. (1989) Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein. *Cell*, **57**, 115–126.
- Weidemann, A., Paliga, K., Durrwang, U., Czech, C., Evin, G., Masters, C.L., and Beyreuther, K. (1997) Formation of stable complexes between two Alzheimer's disease gene products: Presenilin2 and beta-amyloid precursor protein. *Nature Medicine*, **3**, 328–332.
- Wertkin, A.M., Turner, R.S., Pleasure, S.J., Golde, T.E., Younkin, S.G., Trojanowski, J.Q., and Lee, V.M.-Y. (1993) Human neurons derived from a teratocarcinoma cell line express solely the 695-amino acid amyloid precursor protein and produce intracellular β amyloid or A4 peptides. *Proceedings of the National Academy of Science*, **90**, 9513–9517.
- Wiertz, E.J.H.J., Jones, T.R., Sun, L., Bogoy, M., Geuze, H.J., and Ploegh, H.L. (1996a) The human cytomegalovirus US 11 gene product dislocates MHC class 1 heavy chains from the endoplasmic reticulum to the cytosol. *Cell*, **84**, 769–779.
- Wiertz, E.J.H.J., Tortorella, D., Bogoy, M., Yu, J., Mothes, W., Jones, T.R., Rapoport, T.A., and Ploegh, H.L. (1996b) Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature*, **384**, 432–438.
- Wild-Bode, C., Yamazaki, T., Capell, A., Leimer, U., Steiner, H., Ihara, Y., and Haass, C. (1997) Intracellular generation and accumulation of amyloid β -peptide terminating at amino acid 42. *Journal of Biological Chemistry*, **272**, 16085–16088.
- Wisniewski, H.M., Wegiel, J., Wang, K.C., Kujawa, M., and Lach, B. (1989) Ultrastructural studies of the cells forming amyloid fibers in classical plaques. *Canadian Journal of Neurological Sciences*, **16**, 535–542.

- Xia, W., Zhang, J., Perez, R., Koo, E.H., and Selkoe, D.J. (1997) Interaction between amyloid precursor protein and presenilins in mammalian cells: Implications for the pathogenesis of Alzheimer disease. *Proceedings of the National Academy of Science*, **94**, 8208–8213.
- Xu, H., Sweeney, D., Wang, R., Tninkaran, G., Lo, A.C.Y., Sisodia, S.S., Greengard, P., and Gandy, S. (1997) Generation of Alzheimer's β -amyloid protein in the trans-Golgi in the apparent absense of vesicle formation. *Proceedings of the National Academy of Science*, **94**, 3748–3752.
- Yamaguchi, H., Hirai, S., Morimatsu, M., Shoji, M., and Harigaya, Y. (1988) Diffuse type of senile plaques in the brains of Alzheimer-type dementia. *Acta Neuropathologica*, **77**, 113–119.
- Yan, S.D., Fu, J.-F., Soto, C., Zhu, H., Al-Mohanna, F., Collison, K., Zhu, A., 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.
- Yamazaki, T., Haass, C., Saido, T.C., Omura, S., and Ihara, Y. (1997) Specific increase in amyloid β -protein 42 secretion ratio by calpain inhibition. *Biochemistry*, **36**, 8377–8383.
- Zhong, Z., Higaki, J., Murakami, K., Wang, Y., Catalano, R., Quon, D., and Cordell, B. (1994) Secretion of β -amyloid precursor protein involves multiple cleavage sites. *Journal of Biological Chemistry*, **269**, 627–632.

7. **THE CELL BIOLOGY OF AMYLOID PRECURSOR PROTEIN**

BART DE STROOPER and FRED VAN LEUVEN

*Experimental Genetics Group, Center for Human Genetics, Vlaams interuniversitair
Instituut voor Biotechnologie (VIB), K.U.Leuven, Campus Gasthuisberg, B-3000 Leuven*

The study of the cell biology of amyloid precursor protein is driven by the question how its abnormal biochemical processing can cause neurodegeneration. Most investigations have therefore focussed on its metabolism in general and the generation of the amyloid peptide in particular. Other aspects of its cell biology, including its function, have still to be studied in more detail. One clue to the function of amyloid precursor protein is hidden in its fundamental property to traffic throughout the different subcellular compartments of the secretory and the endocytic pathways. This has also implications for its metabolic processing, which can therefore only be understood in the context of (polarized) protein transport in the cell. This will become the main topic of the current chapter. For a more detailed discussion of the function of amyloid precursor protein and of the characteristics of the enzymes (“secretases”) involved in its processing, the reader is referred to the contributions in this volume by Masters, Multhaup and Beyreuther and by Citron and Selkoe.

Biosynthesis, Subcellular Trafficking and Secretase Processing of Amyloid Precursor Protein

Secretory processing of amyloid precursor protein in the biosynthetic pathway

The amyloid precursor protein gene contains 19 exons of which exon 7, 8 and 15 can be alternatively spliced. All possible 8 splice variants have been detected by RT-PCR in tissues (Sandbrink *et al.*, 1994). By consequence at least 8 different amyloid precursor protein core proteins can be synthesized as type I integral membrane proteins in the endoplasmic reticulum. Some of the splice variants contain a Kunitz type of proteinase inhibitor (Tanzi *et al.*, 1988; Ponte *et al.*, 1988; Kitaguchi *et al.*, 1988), and have a function in blood coagulation (Mahdi *et al.*, 1995). Classical N- and O-glycosylation occurs during transit through the endoplasmic reticulum and the Golgi apparatus (Weidemann *et al.*, 1989). The splicing of exon 15 creates a chondroitin sulfate glycosaminoglycan attachment site which is used by astrocytes but not by neurons. The resulting high molecular weight amyloid precursor protein has been called appican (Shioi *et al.*, 1992; Shioi *et al.*, 1995; Pangalos *et al.*, 1995). Addition of sulfate and phosphate in the late Golgi compartment and at the cell surface further increases the structural

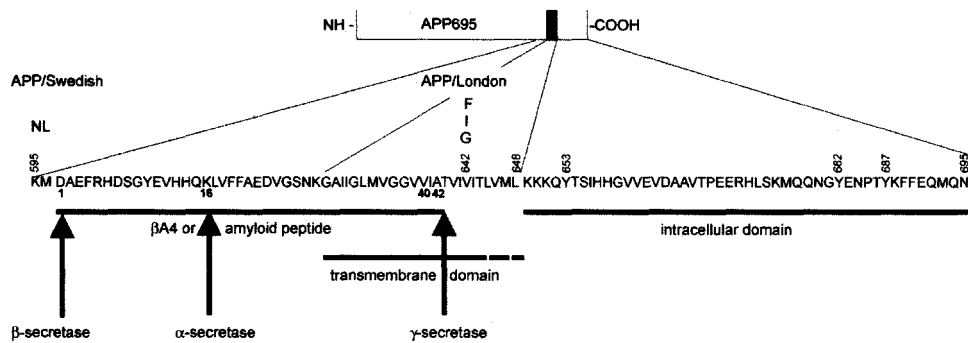


Figure 1. β A4-amyloid, Transmembrane and Cytoplasmic Domain of Amyloid Precursor Protein. The primary amino acid sequence (one letter code) of the carboxyterminal part of APP695 is displayed. The Swedish and London type of clinical mutations and the secretase cleavage sites are indicated. The numbering above the sequence refers to the holoprotein, residue 1 is the first amino acid residue of the signal sequence. The numbering below refers to the amyloid peptide sequence. See text for further details.

complexity of the amyloid precursor proteins (Weidemann *et al.*, 1989; Hung and Selkoe, 1994; Suzuki *et al.*, 1994; Walter *et al.*, 1997). Integral membrane amyloid precursor protein (holo-APP), is cleaved by α -, or β - (and possible γ)-secretases (see the chapter by Citron and Selkoe), which results in the release of the ectodomain of amyloid precursor protein (soluble APP) and the release of β A4-amyloid peptide (Figure 1). Much attention has gone to the question whether holo-APP is cleaved by α -secretase in the Trans-Golgi and the transport vesicles to the cell surface, at the cell surface or in early endosomes after endocytosis (Sisodia *et al.*, 1992; Sambamurti *et al.*, 1992; De Strooper *et al.*, 1992, 1993; Kuentzel *et al.*, 1993; Haass *et al.*, 1995a,b; Refolo *et al.*, 1995). Experiments using proteinase inhibitors, inhibitors of endocytosis, cell surface labeling and cell fractionation, have provided evidence for all three localizations, indicating that the (not yet) identified enzymes responsible for α -secretase cleavage are present in the three compartments (Figure 2). One possibility is therefore that the enzyme(s) responsible for this cleavage cycle between these compartments, similar to other protein processing enzymes like furin. Furin and some related proteinases are however not likely to cleave holo-APP (De Strooper *et al.*, 1995a). Differences in cell type, culture conditions and experimental set up, which all affect the rates of endocytosis and recycling, could explain why in some cells most of the α -secretase processing is detected at the cell surface (Sisodia *et al.*, 1992), while in other cells most is found in an intracellular compartment (Sambamurti *et al.*, 1992). The alternative possibility is that a set of different proteinases is responsible for α -secretase processing. This would explain the heterogeneity of the cleavage sites (Zhong *et al.*, 1994; Simons *et al.*, 1996) and the “relaxed” specificity of the enzyme (Maruyama *et al.*, 1991; Sisodia *et al.*, 1992; De Strooper *et al.*, 1992, 1993). The netto result of cleavage and endocytosis of the remaining holo-APP is the rapid removal of cell surface expressed APP with an estimated half life of less than ten minutes (Koo *et al.*, 1996). Only minor amounts of amyloid precursor protein (as compared to the total cellular pool) are by consequence detected at the cell surface (Sambamurti *et al.*, 1992; Kuentzel *et al.*, 1993). The cleavage of APP by α -secretase results in the generation of soluble APP which might have biological functions in growth regulation and neuroprotection, and, in case of the presence of the Kunitz proteinase inhibitor domain, in blood coagulation (see the chapter by Masters, Multhaup and Beyreuther). The remaining

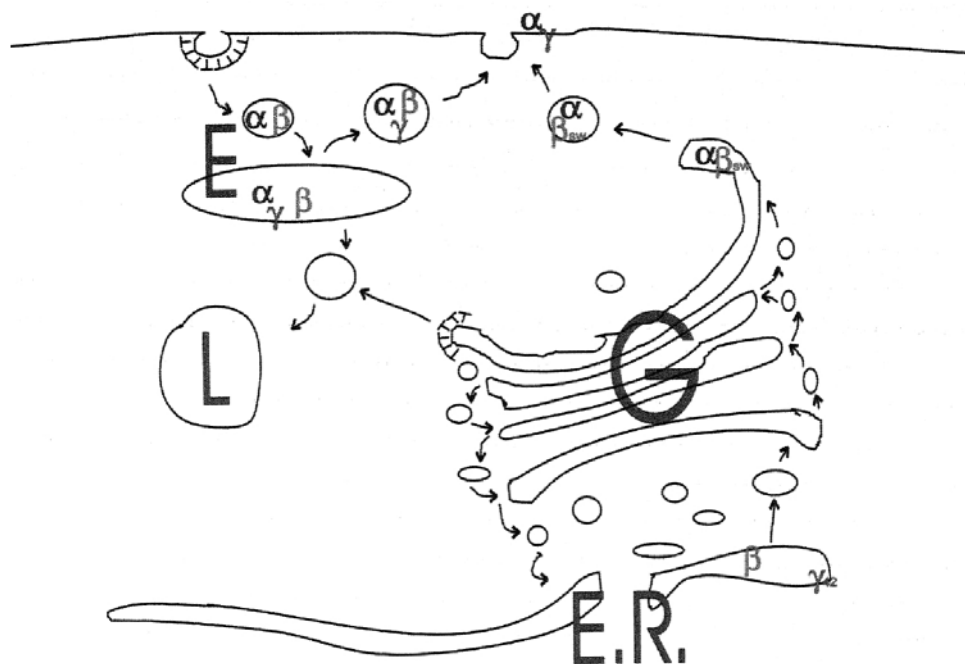


Figure 2. Subcellular Trafficking of Amyloid Precursor Protein. Schematic drawing of the secretory and endocytic pathways followed by APP in the cell. E.R.: endoplasmic Reticulum, G: Golgi apparatus, E: endosomes, L: lysosome. The putative subcellular localization of α -, β -, and γ -secretase activities are indicated. For further discussion and references: see text.

carboxyterminal APP-fragments in the cell membrane have a relative long half life and can be detected to different extent in metabolically labeled cells (Oltersdorf *et al.*, 1990). In conclusion, only a minor fraction of the total amyloid precursor protein pool is thus available for cell biological interactions at the cell surface, which suggests that its main function could be intracellular.

Endocytosis and amyloid peptide generation

Surface iodinated amyloid precursor protein is a precursor to the β A4-amyloid peptide. The generation of this peptide is inhibited when the endocytosis signal-YENPTY-in the cytoplasmic tail of APP is removed (Figure 1). Similarly, when clathrin coat assembly, and therefore endocytosis, is inhibited by culturing cells in low potassium culture conditions, much less amyloid peptide is produced (Koo and Squazzo, 1994; Perez *et al.*, 1996). A slight delay in the production of β A4-peptide compared to the generation of soluble APP is supporting the conclusion that the amyloid peptide is generated after endocytosis of APP holoprotein from the cell surface (Perez *et al.*, 1996). Interestingly, opposite results were obtained with APP containing the Swedish mutation, which makes it a better substrate for β -secretase (see Figure 1): β A4-peptide generation occurs independently from the endocytosis signal, and the kinetics of production parallel those for soluble APP generation (Martin *et al.*, 1995; Schrader-

Fischer and Paganetti, 1996; Perez *et al.*, 1996; Thinakaran *et al.*, 1996). The β -secretase acting on this mutant APP is apparently operational in the Golgi apparatus and the transport vesicles to the cell surface (Figure 2). This is further corroborated by immunocytochemical evidence demonstrating the presence of β -secretase generated amyloid precursor protein cleavage products in these compartments (Haass *et al.*, 1995b; Stephens and Austen, 1996). From these observations, one must conclude that the β -secretase, at least the one that cleaves the Swedish APP mutant, is present in the same compartments as α -secretase, and that in the competition for amyloid precursor protein substrate, α -secretase has the higher affinity for wild type APP and β -secretase for the Swedish type APP (Haass *et al.*, 1995). The other possibility, that a set of different enzymes are functioning as β -secretases (one cleaving wild type APP, the other cleaving Swedish type APP) can however not be ruled out at the moment (Citron *et al.*, 1995). Moreover, in some cell types such as MDCK cells (see below), heterogeneity of the β -secretase cleavage site has been observed, arguing for multiple proteinases capable of cleaving APP at and around the β -secretase cleavage site. Finally, it should be mentioned, that recently evidence for a β -secretase activity in the endoplasmic reticulum and intermediate compartment of neurons has been found (Chyung *et al.*, 1997).

Lysosomal processing of amyloid precursor protein

In PC 12 neuroendocrine cells and in 293 cells more than 70% of newly synthesized amyloid precursor protein is degraded (Caporaso *et al.*, 1992; Knops *et al.*, 1992). This degradation is partially inhibited by chloroquine or ammonium chloride, suggesting the involvement of lysosomes. This was corroborated by experiments using lysosomal proteinase inhibitors, by antibody uptake experiments and by the isolation of lysosomal fractions in the presence and absence of leupeptin, all demonstrating that amyloid precursor protein is metabolized in lysosomes and that an array of carboxyterminal derivatives is produced (Estus *et al.*, 1991; Haass *et al.*, 1992; Golde *et al.*, 1992; Siman *et al.*, 1993; Yamazaki *et al.*, 1996). Parts of these fragments are potentially amyloidogenic because they contain the amyloid sequence. It is not clear whether these fragments are indeed further processed towards amyloid peptide and can contribute to the amyloidogenesis in Alzheimer's brain.

Another unsettled issue is whether all APP that ends up in the lysosomes must first travel over the cell surface. Several indirect arguments have been advanced (Kuentzel *et al.*, 1993; De Strooper *et al.*, 1993; Lai *et al.*, 1995) to suggest that an important fraction of newly synthesized amyloid precursor protein is transported directly from the Trans-Golgi network towards the lysosomes via a clathrin mediated pathway (Figure 2) similar to the mannose-6-phosphate receptors and the lysosome associated membrane glycoproteins (Traub and Kornfeld, 1997).

Cell biology of γ -secretase processing

From a cell biological point of view the γ -secretase cleavage, which releases the carboxyterminal end of the amyloid peptide (Figure 1), raises conceptual problems. The classical idea of the transmembrane domain of APP (extending from Gly625 to Leu648 and ending just before the triplet Lys649–651, implies that this cleavage occurs in the hydrophobic environment of the cell membrane (Figure 1). The possibility that cell membrane damage in Alzheimer's Disease exposes this region to γ -secretase(s) is

not unlikely. However, since amyloid peptide generation occurs also in normal cells without any sign of membrane damage (Haass *et al.*, 1992b; Shoji *et al.*, 1992), a more physiological mechanism must exist. Tischer and Cordell (1996) proposed the interesting idea that the transmembrane domain of APP is actually shorter than predicted on theoretical grounds. They introduced small deletions and charged amino acid residue substitutions in the transmembrane domain at and around the γ -secretase cleavage site and determined a minimal region which did not interfere with the normal processing of APP. This confined the transmembrane region carboxyterminally to amino acid residues Val642 and Ile643. The most carboxyterminal γ -secretase cleavage site at amino acid residues Thr639-Val640 comes then very close to the cytoplasmic side of the membrane, at least at the level of the endoplasmic reticulum membrane which is thinner than the cell membrane. It is conceivable that a cleavage at the cytoplasmic side is sufficient to release the transmembrane peptide into the lumen of the endoplasmic reticulum, where other " γ -secretases" then could act as carboxypeptidases and generate the β A4-peptides ending at residues 40 or 42 (Figure 1). A precursor-product relationship exists between the carboxyterminal fragments of APP generated by α - and β -secretase and the amyloid peptide generation by γ -secretase (Dyrks *et al.*, 1993; Higaki *et al.*, 1995; De Strooper *et al.*, 1998). Since the α - and β -secretases remove most of the hydrophilic ectodomain of APP, it could be envisaged that the remaining transmembrane stub becomes less stably inserted in the membrane, also helping to expose the γ -secretase site to proteinases in the cytoplasm.

Although hypothetical and based on indirect evidence, a coherent picture of amyloid peptide generation in the cell is emerging which accommodates most observations (Figure 2). Two separate pools of amyloid peptide are generated in neurons and in other cell types, one defined as "intracellular", the other as "secreted" (Wertkin *et al.*, 1993; Turner *et al.*, 1996; Tienari *et al.*, 1997; Wild-Bode *et al.*, 1997). The intracellular pool contains relatively more amyloid peptide ending at residue 42, is increased in the presence of brefeldin A (which fuses the Golgi with the endoplasmic reticulum and blocks further transport to the trans-Golgi network), and is not sensitive to inhibitors of endocytosis. This pool of peptide is therefore apparently generated in the endoplasmic reticulum, possibly by mechanisms suggested above. In neurons, amyloid peptide ending at residue 42 was indeed located to the endoplasmic reticulum (K.Beyreuther, personal communication). The second pool is generated in early endosomes or at the cell surface, by the combined activities of β -secretases and (other) γ -secretases generating secreted peptide ending at amino acid 40. The two major γ -secretases activities (yielding β A42 and β A40) are differentially sensitive to calpain proteinase inhibitors (Higaki *et al.*, 1995; Citron *et al.*, 1996; Klafki *et al.*, 1996), arguing that two different enzymes are involved. Mutations in the presenilins, which are mainly located in the endoplasmic reticulum (Walter *et al.*, 1996; De Strooper *et al.*, 1997), increase β A42 peptide production, which fits the hypothesis that γ -secretase generating the 42 peptide is operational at the level of the endoplasmic reticulum membrane.

Trafficking of Amyloid Precursor Protein in Epithelial MDCK Cells

Amyloid lesions in brains of Alzheimer's Disease patients are always associated with polarized cell types, e.g. neurons and endothelial cells. This raises the question whether amyloid precursor protein and its various proteolytic products (soluble APP, β A4, and p3) are secreted in a polarized way, i.e. in neurons to axons or dendrites and in epithelial cells to the apical or basolateral side. Several cell

culture models are available to study this type of problem. The epithelial Madin-Darby canine kidney (MDCK) cell line is by far the best characterized one. The primary culture of hippocampal neurons in combination with recombinant Semliki Forest Virus infection (Dotti and Simons, 1990) provides in the context of Alzheimer's Disease a particular relevant alternative. The neurons are however relatively difficult to grow and to transfect, and studies of protein trafficking to the two functionally distinct domains of axons and dendrites are mainly limited to immunocytochemistry. The MDCK cell line was therefore used to analyze APP sorting and transport at the biochemical level. These cells grow as tight epithelial monolayers on tissue culture filters, separating the apical from the basolateral compartment. The secretion of soluble APP, the β A4- and the p3 peptides in these two compartments, and the arrival of holo-APP at the apical or the basolateral cell surface is followed by appropriate surface labeling techniques and immune precipitation. Since amyloid precursor protein is endogenously expressed by MDCK cells (Haass *et al.*, 1994, De Strooper *et al.*, 1995c), like kidney cells do *in vivo*, the study of this system is physiologically relevant. Moreover, since proteins of the axonal or the dendritic surface in neurons are usually sorted to the apical, respectively the basolateral side in MDCK cells, the sorting mechanisms in neurons and in MDCK cells are at least partially conserved, (Dotti and Simons, 1990, for a review see Banker et Goslin 1994).

The first surprise with amyloid precursor protein in the MDCK system is its strong basolateral orientation: both soluble APP and holo-APP are sorted exclusively to the basolateral compartment (Haass *et al.*, 1994, 1995; Lo *et al.*, 1994; De Strooper *et al.*, 1995c,d). Since APP is transported along axons in neurons (Koo *et al.*, 1990; Ferreira *et al.*, 1993; Sisodia *et al.*, 1993; Simons *et al.*, 1995; Yamazaki *et al.*, 1995), APP does not obey the axonal/apical sorting rule (see below however). Similar studies performed on the basolateral sorting of proteins in MDCK cells, such as the LDL receptor, the asialoglycoprotein receptor, the polymeric immunoglobulin receptor and the Fc-receptor, have demonstrated that short contiguous segments of 10–15 amino acids in the cytoplasmic domains are the only requisite for basolateral targeting (Casanova *et al.*, 1991; Hunziker and Fumey, 1994; Matter *et al.*, 1993; Geffen *et al.*, 1993). These short peptide signals are very similar to tyrosine based endocytic signals that cause many membrane receptors to cluster in coated pits. In addition, bulky hydrophobic amino acid residues or dileucine motifs can mediate basolateral sorting. In the cytoplasmic domain of amyloid precursor protein three candidate tyrosines are present. Two of them, Tyr682 and Tyr687 are positioned close to each other in a clear consensus signal for endocytosis, YENPTY (Figure 1). Deletion of (parts of) the cytoplasmic domain of amyloid precursor protein containing this signal did not perturb its basolateral delivery in MDCK cells, indicating that this region is only involved in endocytosis as discussed above. In contrast, complete deletion of the cytoplasmic domain, or mutation of the third tyrosine at position 653 in the amyloid precursor protein sequence resulted in randomized holo-APP sorting. The secretion of soluble APP remained however basolateral, suggesting that a second basolateral mechanism was operating on soluble APP independently from the cytoplasmic domain (Haass *et al.*, 1995a; De Strooper *et al.*, 1995c). This was further confirmed using truncated versions of APP with translational stop codons at the α -, β -, or γ -secretase cleavage sites. All these soluble forms are secreted into the basolateral compartment, indicating that they contain basolateral sorting information. The integral membrane domain and the amyloid peptide region itself are therefore dispensable for the correct targeting of the ectodomain of APP (in contrast with what was found in neurons, see below). That two independent, but synergistic mechanisms are responsible for the

basolateral sorting of APP, was further confirmed by the differential sensitivity of both mechanisms to alkalinization of intracellular acidic compartments. The secretion of soluble APP, but not the sorting of holo-APP, is completely randomized by ammonium chloride, methylamine or bafilomycin, indicating that the interaction of soluble APP with its sorting mechanism, or the sorting mechanism itself, is pH dependent (De Strooper *et al.*, 1995c; Haass *et al.*, 1995a). Soluble APP is therefore most likely sorted by a similar mechanism as the one acting on other basolaterally secreted soluble proteins (Caplan *et al.*, 1987). Further evidence for two independent sorting mechanisms operating on APP was obtained with a horse radish peroxidase/amyloid precursor protein chimeric protein (De Strooper *et al.*, 1995d). This chimera contains the cytoplasmic domain, the integral membrane domain, and the amyloid region of APP, while the ectodomain is replaced by horse radish peroxidase enzyme. The surface localization of this chimera was basolateral in accordance with the presence of the cytoplasmic domain of APP. The soluble ectodomain, generated by secretase processing, contains, apart from the amyloid region, no APP sequences anymore. It was secreted in a randomized fashion. The results also indicate that α -secretase in MDCK cells acts before holo-APP is inserted in the basolateral vesicles budding from the trans-Golgi network (Figure 2).

The further deciphering of the ectodomain sorting information turned out to be difficult. Deletion of exon 15 from the APP cDNA (which yields a naturally occurring APP splice variant, see above), results in the randomized sorting of the ectodomain. However, basolateral sorting becomes restored with larger deletions in and around exon 15. The basolateral sorting information in the ectodomain of amyloid precursor protein therefore seems to depend on complicated structure/function relationships. Most likely, the conformation of a critical sorting domain, amino terminal from the deleted regions is influenced by the presence or absence of exon 15 (Hartmann *et al.*, 1996). Further research should aim at identifying the molecular components/receptors responsible for both the cytoplasmic and the ectodomain sorting of amyloid precursor protein.

What is, apart from the fundamental cell biological importance, the relevance of these studies for Alzheimer's Disease? First, the Swedish mutation causes abnormal secretion of part of the ectodomain of APP into the apical compartment (Lo *et al.*, 1994; De Strooper *et al.*, 1995c), which suggests that missorting of APP is a potential factor in the pathology of Alzheimer's Disease. It demonstrates at least in principle that abnormal cleavage of APP could interfere with the normal cell biology of APP (De Strooper *et al.*, 1995c). Second, the results establish firmly the strong relationship between amyloid precursor protein processing and subcellular trafficking. This is best illustrated by the fact that in the absence of the cytoplasmic domain of APP, no amyloid peptide starting at Asp597 is produced anymore, while alternative peptides starting 5 amino acid residues more amino terminal and three amino acid residues more carboxyterminal are still produced (Figure 1). Likely, other β -secretase like enzymes are responsible for these cleavages (Haass *et al.*, 1995a). Interestingly, the polarized secretion of the peptides follows mainly the sorting of holo-APP, indicating that their sorting is largely dictated by the cytoplasmic domain of APP. The β - and γ -secretases generating the peptides therefore operate after the sorting of APP in the correct basolateral transport vesicles, and by consequence, after the trans-Golgi network (Figure 2). The observation that the p3 fragment (which is generated by α -secretase, see Citron and Selkoe) remains delivered to the basolateral side, would suggest that the γ -secretase(s) cleaving the α -secretase generated carboxyterminal fragment is mainly operational in the basolateral pathway. To prove this directly will remain difficult until all secretases have been

identified. Finally, the type of studies proposed here are directly relevant for our understanding of the generation of amyloid deposits at the basolateral side of the blood vessels in the brain of Alzheimer's Disease patients.

Transport and Metabolism of Amyloid Precursor Protein in Neurons

While APP is expressed by all cell types studied, amyloid plaques and Alzheimer's Disease are a problem of the brain. The search for brain specific factors has stimulated research on the metabolism of APP in astrocytes, microglia and especially neurons.

What source of neurons should be used

For obvious reasons primary cultures of human (fetal) brain should only be performed under stringent ethical regulations, which limits the practical use of this type of culture. Good *in vitro* alternatives for human neurons are not readily available, since most stable neuronal like cell lines only partial mimic the neuronal phenotype. They do not develop for instance full axonal and dendritic polarity. The NT2N human neuroblastoma cell line is apparently a break-through in this regard (Wertkin *et al.*, 1993; Chyung *et al.*, 1997), although the experience with these cells is still relatively limited. In contrast, the culture of rodent neurons from different anatomical sites of the brain at different stages of development has been established since a long time and is very well characterized. One problem with mouse and rat for the study of Alzheimer's Disease is that they do not develop spontaneously amyloid plaques. This suggests that certain important components of the amyloid plaque generating pathway are lacking in their brains. Mouse and rat APP diverge only in 17 and 18 amino acid residues, respectively, from their human homologue. Three substitutions are located in the amyloid peptide sequence itself (Shivers *et al.*, 1988; Yamada *et al.*, 1987; De Strooper *et al.*, 1991). APP from other species such as monkey, polar bear or dog contain amyloid sequences that are identical to the human sequence (Johnstone *et al.*, 1991; Selkoe *et al.*, 1987). These animals develop amyloid plaques, suggesting the possibility that the few differences in the amyloid sequence are sufficient to "protect" rodents against Alzheimer's Disease. This was directly confirmed in rat hippocampal neurons expressing mouse or human APP (De Strooper *et al.* 1995b). Both proteins were expressed using recombinant Semliki Forest Virus. This expression system has been used successfully before to express heterologous proteins in neurons, and offers the advantage of relatively high protein expression without affecting the polarized organization of the cells during the early phase of infection (Ikonen *et al.*, 1993; De Hoop *et al.*, 1994). For similar levels of expression, three times more amyloid peptide was produced from human than from mouse amyloid precursor protein. Substituting the three amino acid residues in the mouse sequence by their human counterparts was sufficient to increase the amyloid peptide production to the levels obtained with the human form. The single Gly601 Arg substitution, which is in the vicinity of the β -secretase site, was responsible for most of the effect, and it was concluded that this substitution makes mouse amyloid precursor protein a relatively bad substrate for β -secretase (De Strooper *et al.*, 1995b). The finding explains why rodentia do not develop amyloid plaques when aging, in contrast to other species. Functionally important is, that simple "humanization" of the amyloid region in rodentia restores amyloid production. Thus, rodent neurons

must contain the whole subset of secretases involved in the amyloidogenic processing and are therefore useful to study this aspect of the cell biology of amyloid precursor protein. This implies also that neurons derived from mice in which candidate secretases, such as cathepsin D, have been inactivated by homologous recombination, can be used to investigate their role in APP processing (Saftig *et al.*, 1996). Radioactive sequencing of the carboxyterminal fragments generated by secretase processing in rat neurons further substantiated this conclusion (Simons *et al.*, 1996). All cleavages documented before in human cells and brain were confirmed in the rat neurons. Interestingly, heterogeneity at the α -secretase is observed, with fragments starting at residue Leu 17 and at residues Gln 15 and Glu 11 of the amyloid peptide sequence (Figure 1). The first cleavage is at the classical α -secretase site (Esch *et al.*, 1990), the last one is the first residue of β A4(11–42), a major component of Alzheimer's Disease amyloid plaques (Masters *et al.*, 1985, Naslund *et al.*, 1994). This was interpreted as suggesting that a set of enzymes are involved in α -secretase processing, in agreement with conclusions from studies in the other cell types discussed above. The picture of an anti-amyloidogenic single α -secretase cleavage of amyloid precursor protein in the default secretory pathway, is clearly an oversimplification of reality. The third important conclusion from these studies is that “humanizing” of the mouse amyloid precursor protein gene should yield a model to study the pathogenesis of sporadic Alzheimer's disease. Such mice could probably be used to evaluate the effect of environmental factors on amyloidogenesis. A “humanized” mouse, containing the Swedish mutation, was developed recently (Reaume *et al.*, 1996). In contrast to other models, the mutated amyloid precursor protein is driven by the endogenous promotor which results in normal levels and patterns of expression in tissues and cells.

Processing of amyloid precursor protein in neurons

Several particularities of the processing of amyloid precursor protein in astrocytes, microglia and neurons, as compared to established cell lines such as CHO, kidney 293, and COS-cells, have been found. Differences in the levels of α -secretase processing are most likely explained by the culture conditions of neurons and astrocytes used for these experiments (Haass *et al.*, 1991; Hung *et al.*, 1992; Allinquant *et al.*, 1994; Simons *et al.*, 1996; LeBlanc *et al.*, 1996, 1997). For instance more α -secretase activity was observed in hippocampal neurons that elaborate contacts *in vitro* than in cervical sympathetic ganglion neurons, which do not (Simons *et al.*, 1996). This is in agreement with the notion that α -secretase activity is tightly regulated by second messengers (see for a detailed discussion the chapter written by Nitsch *et al.*) Of importance for the study of Alzheimer's Disease, is the strong amyloidogenic nature of APP processing in neurons. Both human neurons and rodent neurons expressing human amyloid precursor protein, have a strong tendency to produce amyloid peptide and intracellular amyloidogenic fragments in comparison to microglia, astrocytes or fibroblasts. Neurons are apparently the major contributor to the amyloid plaques in the brain (De Strooper *et al.*, 1995; Simons *et al.*, 1996; LeBlanc *et al.*, 1997; Zhao *et al.*, 1996; but see also Busciglio *et al.*, 1993). Neurons produce also an array of carboxyterminal fragments that are relatively stable, since easily detected in the absence of lysosomal inhibitors (De Strooper *et al.*, 1995; Amaratunga *et al.*, 1995; Simons *et al.*, 1996; LeBlanc *et al.*, 1996, 1997). Such fragments could be involved in neurotoxicity (Yoshikawa *et al.*, 1992; Fukuchi *et al.*, 1993; Fraser *et al.*, 1997). Interestingly, clinical mutations that cause increased β -amyloid peptide secretion, also cause changes in the spectrum of intracellular

carboxyterminal precursor protein fragments, with a shift to longer forms (De Strooper *et al.*, 1995). Finally, neurons contain a detectable pool of intracellular amyloid peptide (Wertkin *et al.*, 1993; Turner *et al.*, 1996; Tienari *et al.*, 1997). Whether this pool is a consequence of the much more complicated trafficking pathways of amyloid precursor protein in neurons than in other cell types, and whether this intracellular β A4-amyloid peptide pool is involved in the initial stages of Alzheimer's Disease remain to be established. Given the brain specificity of Alzheimer's Disease, this particular aspect of amyloid precursor protein metabolism deserves further study.

In conclusion, all available data support the idea of a central role of neurons in amyloid production in Alzheimer's Disease (Figure 3). High concentrations of aggregated amyloid peptide are toxic for neurons (Yankner *et al.*, 1990; Pike *et al.*, 1993), explaining neurodegeneration in the more advanced stages of Alzheimer's Disease. Low concentrations of (soluble) amyloid peptide on the other hand increase the vulnerability of neurons to apoptosis by downregulating Bcl-2 and up regulating Bax expression (Paradis *et al.*, 1996). This induces abnormal processing of APP and amyloid peptide production by neurons (LeBlanc, 1995), creating a positive feed back loop. In addition, the change in the spectrum of carboxyterminal fragments as the logical correlative of any change in β A4-peptide secretion (De Strooper *et al.*, 1995) could also cause apoptosis (Yoshikawa *et al.*, 1992; Fukuchi *et al.*, 1993; Fraser *et al.*, 1997) possibly by influencing intracellular signaling cascades as was found for the APP/London clinical mutation (Okamoto *et al.*, 1996; Yamatsuji *et al.*, 1996; Gianbarella *et al.*, 1997). This creates an additional positive feed back loop, leading to the progressive spreading of the disease.

Polarized trafficking of amyloid precursor protein in neurons

The trafficking of APP in neurons with their long axonal and dendritic extensions is of particular interest. Several questions are related to this, i.e. whether soluble APP, amyloid peptide and other soluble derivatives of amyloid precursor protein are secreted at the axonal or the dendritic surfaces, and whether amyloid can be taken up and transcytosed by neurons. As mentioned already above, the major technical obstacle is the absence of a physical barrier between axonal and dendritic domains in primary cultures of neurons. Although Campenot chambers (Campenot, 1977) could theoretically help to circumvent this problem, the low amounts of cell material obtained from such cultures precludes further detailed biochemical analyses.

Initial studies demonstrated the accumulation of amyloid precursor protein in rat sciatic nerve axons proximal to a ligation site. The time course of this process indicated that amyloid precursor protein was transported by the fast component of axonal transport (Koo *et al.*, 1990; Sisodia *et al.*, 1993). In hippocampal neurons and cervical ganglia cells similar axonal polarity of amyloid precursor protein was demonstrated (Ferreira *et al.*, 1993; Yamazaki *et al.*, 1995; Simons *et al.*, 1995). Interestingly, the latter two authors found that monoclonal antibodies raised against the ectodomain of amyloid precursor protein are transported retrogradely from the axon to the dendrites, suggesting the transcytosis of APP in neurons. This implies that APP can bind (unknown) ligands at presynaptic sites and transfer them back to the cell body and dendrites, much in the same way as the monoclonal antibodies used in these studies. The functional implications are discussed elsewhere in this volume by Masters, Multhaup and Beyreuther. Amyloid precursor protein is found in clathrin coated pits together with recycling synaptic markers, such as synaptophysin, synaptotagmin and SV2 (Ferreira *et al.*, 1993;

Marquez-Sterling *et al.*, 1997), confirming endocytosis of amyloid precursor protein together with synaptic vesicle components at the presynaptic site. However, since synaptic vesicles, isolated by classical fractionation or by immunoisolation using antisynaptophysin monoclonal antibodies, do not contain APP, APP must be sorted away from the synaptic components once endocytosis has occurred. Since APP is enriched in rab5 positive immunoisolated vesicles, it appears that sorting events occur in a rab5 positive, early endosomal compartment, separating APP from the recycling synaptic vesicles. These results were further corroborated by electron microscopical studies, showing the presence of APP and rab5 on large multilamellar vesicles (Marquez-Sterling *et al.*, 1997; Ikin *et al.*, 1996). This compartment could therefore be the first station in the transcytotic pathway of APP.

As discussed, APP does not obey the axonal/apical and dendritic/basolateral sorting rule as defined by Dotti and Simons (1990). This raises the possibility that the basolateral sorting mechanisms operating on APP in the MDCK cell system, are not existing in neurons or, alternatively, that the axonal sorting mechanism in neurons is overriding the dendritic sorting signals in APP. The Semliki Forest Virus expression system is invaluable for this type of study. It allows generating a pulse of newly synthesized protein by simple infection of the neurons. The transport of this pool of APP can be followed by fixing the neurons at different time points post infection and by analyzing the distribution of APP using fluorescent antibodies against the introduced myc-tag (Simons *et al.*, 1995). Deletion of the cytoplasmic domain or large parts of the luminal/extracellular domain of APP did not alter its axonal transport, while small deletions in the amyloid peptide region resulted in somatodendritic sorting (Tienari *et al.*, 1996). Tunicamycin treatment or deletion of the carbohydrate containing domain caused also missorting, suggesting that carbohydrate is involved in the interaction of APP with the axonal transport machinery. The observations indicate that the axonal sorting information is contained in the carbohydrate and amyloid peptide regions (exons 11–15 and 16–17) in the ectodomain of APP. Since the amyloid region is the less conserved region between mouse and human APP (see above), and since mouse APP is also transported axonally (De Strooper *et al.*, 1995), the specificity of the axonal sorting system involved is apparently relatively relaxed. The possibility that the amyloid region binds other molecules such as glycolipids (Yanagisawa *et al.*, 1995) that could serve as adaptors between APP and the axonal sorting machinery, is one possibility. Glycolipid interactions are indeed believed to be involved in axonal/apical sorting events (Harder and Simons, 1997). In any event, the amyloid peptide region is clearly available for all type of molecular interactions, among them all secretase processing steps. Regulation of trafficking of amyloid precursor protein by proteolytic processing is therefore envisaged. Indirect evidence for this possibility was obtained by expressing the soluble ectodomain of APP in neurons, which is transported to the dendrites (Tienari *et al.*, 1996). α -secretase processing thus destroys the axonal sorting information in APP and releases dendritic/basolateral sorting information in its ectodomain. The basolateral/dendritic sorting rule becomes then again valid.

In conclusion, a consistent (but still hypothetical) picture of the sorting of APP arises. Polarized trafficking of APP in neurons depends on a hierarchy of sorting information in the ecto- and the cytoplasmic domain. The axonal information is dominant when newly synthesized APP is included in axonal transport vesicles in the trans-Golgi network, and becomes inactivated once the synaptic region is reached. The cytoplasmic basolateral/ endocytosis sorting signals become then dominant, redirecting APP to a rab5 compartment, from where the protein is further transcytosed towards the dendrites.

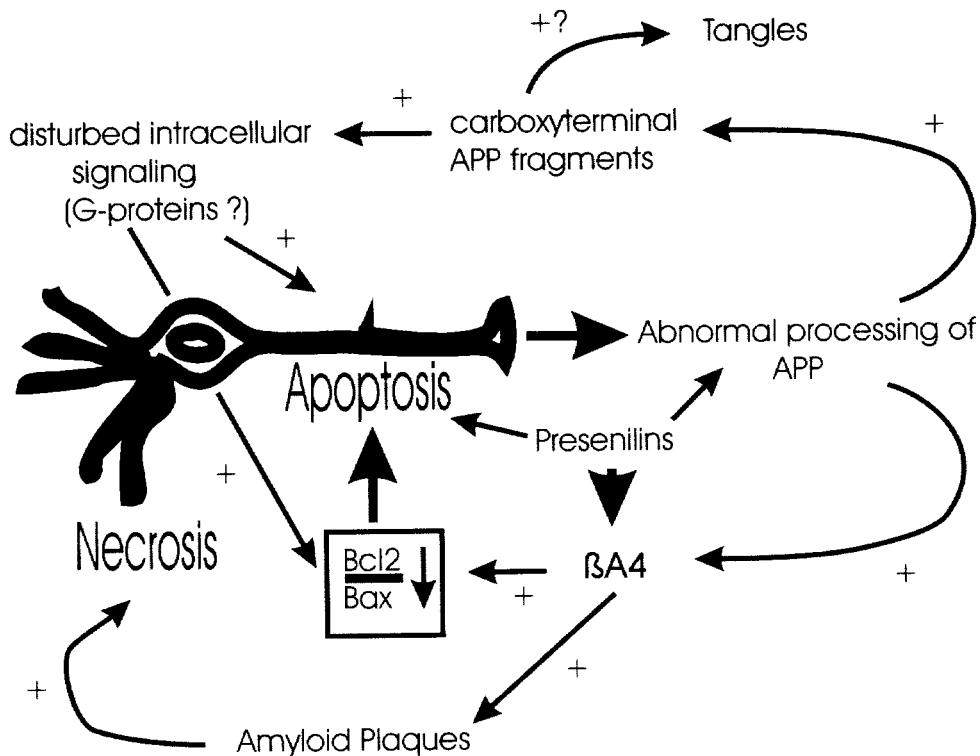


Figure 3. The Central Role of the Neurons in the Progression of Alzheimer's Disease.

Since the recessive basolateral/somatodendritic sorting signal in the ectodomain becomes only operational after cleavage, its functionality depends on the subcellular localization of α -secretase. Whether and how the other sorting information could be regulated is unknown.

REFERENCES

- Allinquant, B., K.L. Moya, C.Bouillot and A.Prochiantz (1994) Amyloid Precursor Protein in cortical neurons: coexistence of two pools differentially distributed in axons and dendrites and association with cytoskeleton. *J. Neurosci.*, **14**, 6842–6854.
- Amaratunga, A., and R.E.Fine (1995) Generation of amyloidogenic C-terminal fragments during rapid axonal transport *in vivo* of β -Amyloid Precursor Protein in the optic nerve. *The Journal of Biological Chemistry*, **270**, 17268–17272.
- Busciglio, J., D.H. Gabuzda, P. Matsudaira and B.A. Yankner (1993) Generation of β -amyloid in the secretory pathway in neuronal and nonneuronal cells. *Proc. Natl. Acad. Sci.*, **90**, 2092–2096.
- Campenot, R.B. (1977) Local control of neurite development by nerve growth factor. *Proc. Natl. Acad. Sci.*, **74**, 4516–4519.

- Caplan, M.J., J.L. Stow, A.P. Newman, J. Madri, H.C. Anderson, M.G. Farquhar, G.E. Palade and J.D. Jamieson (1987) Dependence on pH of polarized sorting of secreted proteins. *Nature*, **15329**, 632–635.
- Caporaso, G.L., S.E. Gandy, J.D. Buxbaum and P. Greengard (1992) Chloroquine inhibits intracellular degradation but not secretion of Alzheimer β /A4 amyloid precursor protein. *Proc. Natl. Acad. Sci.*, **89**, 2252–2256.
- Casanova, I.E., G. Apodaca, and K.E. Mostov (1991) An autonomous signal for basolateral sorting in the cytoplasmic domain of the polymeric immunoglobulin receptor. *Cell*, **66**, 65–75.
- Chyung, S.C., B.D. Greenberg, D.G. Cook, R.W. Doms and V.M.-Y. Lee (1997) Novel β -secretase cleavage of β -Amyloid Precursor Protein in the endoplasmic reticulum/intermediate compartment of NT2N cell. *The Journal of Cell Biology*, **138**, 671–680.
- Citron, M., D.B. Teplow and D.J. Selkoe (1995) Generation of Amyloid β Protein from its Precursor is sequence specific. *Neuron*, **14**, 661–670.
- Citron, M., T.S., Diehl, G. Gordon, A.L. Biere, P. Seubert, and D. Selkoe (1996) Evidence that the 42- and 40-amino acid forms of amyloid protein are generated from the β -amyloid precursor protein by different protease activities. *Proceedings of the National Academy of Sciences*, **93**, 13170–13175.
- Craig, A.M., and G. Banker (1994) Neuronal polarity. *Annual Review of Neuroscience*, **17**, 267–310.
- De Hoop, M., C. von Poser, C. Lange, E. Ikonen, W. Hunziker, and C.G. Dotti (1995) Intracellular routing of Wild-type and Mutated polymeric Immunoglobulin receptor in hippocampal neurons in culture. *The Journal of Cell Biology*, **130**, 1447–1459.
- De Strooper, B., F. Van Leuven and H. Van den Berghe (1991) The amyloid β protein precursor or proteinase nexin II from mouse is closer related to its human homolog than previously reported. *Biochimica et Biophysica Acta*, **1129**, 141–143.
- De Strooper, B., F. Van Leuven and H. Van den Berghe (1992) α 2-Macroglobulin and other proteinase inhibitors do not interfere with the secretion of Amyloid Precursor Protein in mouse neuroblastoma cells. *FEBS Letters*, **308**, 1, 50–53.
- De Strooper, B., L. Umans, F. Van Leuven and H. Van den Berghe (1993) Study of the synthesis and secretion of normal and artificial mutants of murine Amyloid Precursor Protein: cleavage of APP occurs in a late compartment of the default secretion pathway. *The Journal of Cell Biology*, **121**, 295–304.
- De Strooper, B., J.W.M. Creemers, D. Moechars, D. Huylebroeck, W.J.M. Van De Ven, F. Van Leuven and H. Van den Berghe (1995a) Amyloid Precursor Protein is not processed by furin, PACE 4, PC1/3, PC2, PC4 and PC5/6 of the furin family of proprotein processing enzymes. *Biochimica et Biophysica Acta*, **1246**, 185–188.
- De Strooper, B., M. Simons, G. Multhaup, F. Van Leuven, K. Beyreuther and C.G. Dotti (1995b) Production of intracellular amyloid containing fragments in hippocampal neurons expressing mutants of human Amyloid Precursor Protein causing Alzheimer's disease and protection against amyloidogenesis by subtle amino acid substitutions in the rodent sequence. *The EMBO Journal*, **14**, 4932–4938.
- De Strooper, B., K. Craessaerts, I. Dewachter, D. Moechars, B. Greenberg, F. Van Leuven and H. Van den Berghe (1995c) Basolateral secretion of Amyloid Precursor Protein in MDCK cells is disturbed by alterations of intracellular pH and by introducing a mutation associated with familial Alzheimer's disease. *The Journal of Biological Chemistry*, **270**, 4058–4065.
- De Strooper, B., K. Craessaerts, F. Van Leuven and H. Van den Berghe (1995d) Exchanging the extracellular domain of Amyloid Precursor Protein for horse radish peroxidase does not interfere with α -secretase cleavage of the β -amyloid region but randomizes secretion in MDCK cells. *The Journal of Biological Chemistry*, **270**, 30310–30314.

- De Strooper, B., M.Buellens, B.Contreras, L.Levesque, K.Craessaerts, B.Cordell, D.Moechars, M.Bollen, P.Fraser, P.St.George-Hyslop, and F.Van Leuven. (1997) Phosphorylation, subcellular localization, and membrane orientation of the Alzheimer's Disease-associated Presenilins. *The Journal of Biological Chemistry*, **272**, 3590–3598.
- De Strooper, B., P.Saftig, K.Craessaests, H.Venderstichele, G.Guhde, W.Annaert, K.von Figura and F.Van Leuven. Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature*, **391**, 387–390.
- Dotti, C.G., and K.Simons (1990) Polarized sorting of viral glycoproteins to the axon and dendrites of hippocampal neurons in culture. *Cell*, **62**, 63–72.
- Dyrks, T., E.Dyrks, U.Mönnig, B.Urmoneit, J.Turner, and K.Beyreuther (1993) Generation of β A4 from the amyloid precursor protein and fragments thereof. *FEBS Letters*, **335**, 89–93.
- Esch, F.E., P.S.Keim, E.C.Beattie, R.W.Blacher, A.R.Culwell, T.Oltersdorf, D.McClure and P.J.Ward (1990) Cleavage of amyloid β peptide during constitutive processing of its precursor. *Science*, **248**, 1122–1124.
- Estus, S., T.E.Golde, T.Kunishita, D.Blades, D.Lowery, M.Eisen, M.Usiak, X. Qu, T.Tabira, B.D.Greenberg, and S.G.Youngkin (1991) Potentially amyloidogenic, carboxyl-terminal derivatives of the Amyloid Precursor Protein. *Science*, **255**, 726–728.
- Ferreira, A., A.Caceres, and K.Kosik (1993) Intraneuronal compartments of the Amyloid Precursor Protein. *The Journal of Neuroscience*, **13**, 3112–3123.
- Fraser, S.P., Y.-H.Suh, and M.B.A.Djamgoz (1997) Ionic effects of the Alzheimer's Disease β -amyloid precursor protein and its metabolic fragments. *Trends in the Neurosciences* **20**, 67–72.
- Fukuchi, K.-I., B.Sopher, C.E.Furlong, A.C.Smith, N.T.Dang, and G.M.Martin (1993) Selective neurotoxicity of COOH-terminal fragments of the β -amyloid precursor protein. *Neuroscience letters* **154**, 145–148.
- Geffen, I., C.Fuhrer, B.Leitinger, M.Weiss, K.Huggel, G.Griffiths, and M.Spiess (1993) Related signals for endocytosis and basolateral sorting of the asialoglycoprotein receptor. *J. Biol. Chem.*, **268**, 20772–20777.
- Giambarella, U., T.Yamatsuji, T.Okamoto, T.Matsui, T.Ikezu, Y.Murayama, M.A.Levine, A.Katz, N.Gautam, and I.Nishimoto (1997) G protein bg complex-mediated apoptosis by familial Alzheimer's Disease mutant of APP. *EMBO Journal*, **16**, 4897–4907.
- Golde, T.E., S.Estus, L.H.Youngkin, D.J.Selkoe, and S.G.Youngkin (1991) Processing of the Amyloid Protein Precursor to potentially amyloidogenic derivatives. *Science*, **255**, 728–730.
- Haass, C., A.Y.Hung and D.J.Selkoe (1991) Processing of β -Amyloid Precursor Protein in microglia and astrocytes favors an internal localization over constitutive secretion. *The Journal of Neuroscience*, **11**, 3783–3793.
- Haass, C., E.H.Koo, A.Mellon, A.Y.Hung, and D.J.Selkoe (1992a) Targeting of cell-surface β -Amyloid Precursor Protein to lysosomes: alternative processing into amyloid-bearing fragments. *Nature*, **357**, 500–502.
- Haass, C., M.G.Schlossmacher, A.Y., Hung, C.Vigo-Pelfrey, A.Melton, B.L.Ostaszewski, I. Lieberburg, E.H.Koo, D.Schenk, D.B.Teplow, and D.J.Selkoe (1992b) Amyloid β -peptide is produced by cultured cell during normal metabolism. *Nature*, **359**, 322–325.
- Haass, C., E.H.Koo, D.B.Teplow, and D.J.Selkoe (1994) Polarized secretion of β -Amyloid Precursor Protein and amyloid β -peptide in MDCK cells. *Prod. Natl. Acad. Sci.*, **91**, 1564–1568.
- Haass, C., E.H.Koo, A.Capell, D.B.Teplow, and D.J.Selkoe (1995a) Polarized sorting of β -Amyloid Precursor Protein and its proteolytic products in MDCK cells is regulated by two independent signals. *The Journal of Cell Biology*, **128**, 537–547.

- Haass, C., C.A.Lemere, A.Capell, M.Citron, P.Seubert, D.Schenk, L.Lannfelt and D.J.Selkoe (1995b) The Swedish mutation causes early-onset Alzheimer's disease by β -secretase cleavage within the secretory pathway. *Nature Medicine*, **1**, 1291–1296.
- Harder, T., and K.Simons Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. (1997) *Current opinion in Cell Biology*, **9**, 534–542.
- Hartmann, T., C.Bergsdorf, R.Sandbrink, P.J.Tienari, G.Multhaup, N.Ida, S.Bieger, T.Dyrks, A.Weidemann, C.L.Masters, and K.Beyreuther (1996) Alzheimer's Disease β A4 protein release and Amyloid Precursor Protein sorting are regulated by alternative splicing. *The Journal of Biological Chemistry*, **271**, 13208–13214.
- Higaki, J., D.Quon, Z.Zhong, and B.Cordell (1995) Inhibition of β -amyloid formation identifies proteolytic precursors and subcellular site of catabolism. *Neuron* **14**, 651–659.
- Hung, A.Y., E.H.Koo, C.Haass, and D.J.Selkoe (1992) Increased expression of β -Amyloid Precursor Protein during neuronal differentiation is not accompanied by secretory cleavage. *Proc. Natl. Acad. Sci.*, **89**, 9439–9443.
- Hung, A.Y., and D.J.Selkoe (1994) Selective ectodomain phosphorylation and regulated cleavage of β -Amyloid Precursor Protein. *The EMBO Journal*, **13**, 534–542.
- Hunziker, W., and C.Fumey (1994) A di-leucine motif mediates endocytosis and basolateral sorting of macrophage IgG Fc receptors in MDCK cells. *EMBO J.*, **13**, 2963–2967.
- Ikin, A.F., W.G.Annaerts, K.Takei, P.De Camilli, R.Jahn, P.Greenhard, and J.D.Buxbaum (1996) Alzheimer Amyloid Protein Precursor is localized in nerve terminal preparations to Rab5-containing vesicular organelles distinct from those implicated in the synaptic vesicle pathway. *The Journal of Biological Chemistry*, **271**, 31783–31786.
- Ikonen, E., R.G.Parton, W.Hunziker, K.Simons, and C.G.Dotti (1993) Transcytosis of the polymeric immunoglobulin receptor in cultured hippocampal neurons. *Current Biology*, **3**, 635–644.
- Johnstone, E.M., M.O.Chaney, F.H.Norris, R.Pascual, and S.P.Little (1991) Conservation of the sequence of the Alzheimer's disease amyloid peptide in dog, polar bear and five other mammals by cross-species polymerase chain reaction analysis. *Molecular Brain Research*, **10**, 299–305.
- Kitaguchi, N., Y.Takahashi, Y.Tokushima, S.Shiojiri, and I.Hirataka (1988) Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity. *Nature*, **331**, 530–532.
- Knops, J., I.Lieberburg, and S.Sinha (1992) Evidence for a nonsecretory, acidic degradation pathway for Amyloid Precursor Protein in 293 cells (identification of a novel, 22-kDa, β -peptide-containing intermediate). *The Journal of Biological Chemistry*, **267**, 16022–16024.
- Klafki, H.W., D.Abramowski, R.Swoboda, P.A.Paganetti, and M.Staufenbiel (1996) The carboxyterminal termini of β -amyloid peptides 1–40 and 1–42 are generated by distinct γ -secretase activities. *The Journal of Biological Chemistry*, **271**, 28655–28659.
- Koo, E.H., S.S.Sisodia, D.R.Archer, M.L.J., A.Weidemann, K.Beyreuther, P.Fischer, C.L.Masters, and D.L.Price (1990) Precursor of amyloid protein in Alzheimer disease undergoes fast anterograde axonal transport. *Proc. Natl. Acad. Sci.*, **87**, 1561–1565.
- Koo, E.H., and S.L.Squazzo (1994) Evidence that production and release of amyloid β -protein involves the endocytic pathway. *The Journal of Biological Chemistry*, **269**, 17386–17389.
- Koo, E.H., S.L.Squazzo, D.J.Selkoe, and C.H.Koo (1996) Trafficking of cell-surface amyloid β -protein precursor (secretion, endocytosis and recycling as detected by labeled monoclonal antibody). *Journal of Cell Science*, **109**, 991–998.

- Kuentzel, S.L., S.M.Ali, R.A.Altman, B.D.Greenberg, and T.J.Raub (1993) The Alzheimer β -amyloid protein precursor/ protease nexin-II is cleaved by secretase in a trans-Golgi secretory compartment in human neuroglioma cells. *Biochem. J.*, **295**, 367–378.
- Lai, A., S.S.Sisodia, and I.S.Trowbridge (1995) Characterization of sorting signals in the β Amyloid Precursor Protein cytoplasmic domain. *The Journal of Biological Chemistry*, **270**, 3565–3573.
- LeBlanc, A. (1995) Increased production of 4 kDa Amyloid β Peptide in serum deprived human primary neuron cultures: possible involvement of apoptosis. *The Journal of Neuroscience*, **15**, 7837–7846.
- LeBlanc, A., R.Xue, and P.Gambetti (1996) Amyloid Precursor Protein metabolism in primary cell cultures of neurons, astrocytes and microglia. *Journal of Neurochemistry*, **66**, 2300–2310.
- LeBlanc, A., M.Papadopoulos, C.Bélair, W.Chu, M.Crosato, J.Powell and C.G.Goodyer (1997) Processing of Amyloid Precursor Protein in human primary neuron and astrocyte cultures. *Journal of Neurochemistry*, **68**, 1183–1190.
- Lo, A.C.Y., G.Thinakaran, H.H.Slunt, and S.S.Sisodia (1995) Metabolism of the Amyloid Precursor-like Protein 2 in MDCK cells (polarized trafficking occurs independent of the chondroitin sulfate glycosaminoglycan chain). *The Journal of Biological Chemistry*, **270**, 12641–12645.
- Mahdi, F., W.E.Van Nostrand and A.H.Schmaier (1995) Protease Nexin-2/ Amyloid β Protein Precursor inhibits factor Xa in the prothrombinase complex. *The Journal of Biological Chemistry*, **270**, 23468–23474.
- Marquez-Sterling, N.R., A.C.Y.Lo, S.S.Sisodia, and E.H.Koo (1997) Trafficking of cell-surface β Amyloid Precursor Protein: evidence that a sorting intermediate participates in synaptic vesicle recycling. *The Journal of Neuroscience*, **17**, 140–151.
- Martin, B.L., G.Schrader-Fischer, J.Busciglio, M.Duke, P.Paganetti, and B.A.Yankner (1995) Intracellular accumulation of β Amyloid in cells expressing the Swedish mutant Amyloid Precursor Protein. *The Journal of Biological Chemistry*, **270**, 26727–26730.
- Maruyama, K., F.Kemtani, M.Usami, W.Yamao-Harigaya, and K.Tanaka (1991) “Secretase”, Alzheimer amyloid protein precursor secreting enzyme is not sequence-specific. *Biochemical and biophysical research communications*, **179**, 1670–1676.
- Masters, C.L., G.Multhaup, G.Simms, J.Pottgiesser, R.N.Martins and K.Beyreuther (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 Journal*, **4**, 2757–2763.
- Matter, K., J.A.Whitney, E.M.Yamamoto and I.Mellman (1993) Common signals control low density lipoprotein receptor sorting in endosomes and the Golgi complex of MDCK cells. *Cell*, **74**, 1053–1064.
- Naslund, J., E.Schierhorn, H.U., L.Lannfelt, A.D.Roses, L.O.Tjernberg, J.Silberring, S.E.Gandy, B.Winblad, P.Greengard, G.Nordstedt, and L.Teranius (1994) Relative abundance of Alzheimer a beta amyloid peptide variants in Alzheimer Disease and normal aging. *Proc. Natl. Acad. Sci.*, **91**, 9378–9382.
- Okamoto, T., S.Takeda, U.Giambarella, Y.Murayama, T.Matsui, T.Katada, Y.Matsuura, and I. Nishimoto (1996) Intrinsic signaling function of APP as a novel target of three V642 mutations linked to familial Alzheimer’s disease. *The EMBO Journal*, **15**, 3769–3777.
- Oltersdorf, T., P.J.Ward, T.Henriksson, E.C.Beattie, R.Neve, I.Lieberburg, and L.C.Fritz (1990) The Alzheimer Amyloid Precursor Protein (identification of a stable intermediate in the biosynthetic/ degradative pathway. *The Journal of Biological Chemistry*, **265**, 4492–4497.
- Pangalos, M.N., S.Efthimiopoulos, J.Shioi, and K.N.K. (1995) The chondroitin sulfate attachment site of appican is formed by splicing out exon 15 of the Amyloid Precursor gene. *The Journal of Biological Chemistry*, **270**, 10388–10391.

- Paradis, E., H.Douillard, M.Koutroumanis, C.Goodyer, and A.LeBlanc (1996) Amyloid β Peptide of Alzheimer's disease downregulates Bcl-2 and upregulates Bax expression in human neurons. *The Journal of Neuroscience*, **16**, 7533–7539.
- Perez, R.G., S.L.Squazzo, and E.H.Koo (1996) Enhanced release of Amyloid β Protein from codon 670/671 "Swedish" mutant β Amyloid Precursor Protein occurs in both secretory and endocytic pathways. *The Journal of Biological Chemistry*, **271**, 9100–9107.
- Pike, C.J., D.Burdick, A.J.Walencewicz, C.G.Glabe, and C.W.Cotman (1993) Neurodegeneration induced by β Amyloid Peptides *in vitro*: the role of peptide assembly state. *The Journal of Neuroscience*, **13**, 1676–1687.
- Ponte, P., P.Gonzales-DeWhitt, J.Schilling, J.Miller, D.Hsu, B.Greenberg, K.Davis, W.Wallace, I.Lieberburg, F.Fuller, and B.Cordell (1988) A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors. *Nature*, **331**, 525–527.
- Reaume, A.G., D.S.Howland, S.P.Trusko, M.J.Savage, D.M.Lang, B.D.Greenberg, R.Siman, and R.W.Scott (1996) Enhanced amyloidogenic processing of the β Amyloid Precursor Protein in gene-targeted mice bearing the Swedish familial Alzheimer's disease mutations and a "humanized" A β sequence. *The Journal of Biological Chemistry*, **271**, 23380–23388.
- Refolo, L.M., K.Sambamurti, S.Efthimiopoulos, M.A.Pappolla, and N.K.Robakis (1995) Evidence that secretase cleavage of cell surface Alzheimer Amyloid Precursor occurs after normal endocytic internalization. *Journal of Neuroscience Research*, **40**, 694–706.
- Sambamurti, K., J.Shioi, J.P.Anderson, M.A.Pappolla, and N.K.Robakis (1992) Evidence for intracellular cleavage of the Alzheimer's Amyloid Precursor in PC 12 cells. *Journal of Neuroscience Research*, **33**, 319–329.
- Sandbrink, R., C.L.Masters, and K.Beyreuther (1994) β A4-Amyloid Precursor Protein mRNA isoforms without exon 15 are ubiquitously expressed in rat tissues including brain, but not in neurons. *The Journal of Biological Chemistry*, **269**, 1510–1517.
- Saftig, P., C.Peters, K.von Figura, K.Craessaerts, F.Van Leuven, and B.De Strooper (1996) Amyloidogenic processing of Human Amyloid Precursor Protein in Hippocampal Neurons devoid of Cathepsin D. *The journal of Biological Chemistry*, **271**, 27241–27244.
- Schrader-Fischer, G., M.Staufenbiel, and P.A.Paganetti (1997) Insertion of lysosomal targeting sequences to the Amyloid Precursor Protein reduces secretion of β A4. *Journal of Neurochemistry*, **68**, 1571–1580.
- Selkoe, D.J., D.S.Bell, M.B.Podlisny, D.L.Price, and L.C.Cork (1987) Convention of brain amyloid proteins in aged mammals and humans with Alzheimer's Disease. *Science*, **235**, 873–877.
- Shioi, J., J.P.Anderson, J.A.Ripellino, and N.K.Robakis (1992) Chondroitin sulfate proteoglycan form of the Alzheimer's β -Amyloid Precursor. *The Journal of Biological Chemistry*, **267**, 13819–13822.
- Shioi, J., M.N.Pangalos, J.A.Ripellino, D.Vassilacopoulou, C.Mytilineou, R.U.Margolis, and N.K.Robakis (1995) The Alzheimer amyloid precursor proteoglycan (appican) is present in brain and is produced by astrocytes but not by neurons in primary neural cultures. *The Journal of Biological Chemistry*, **270**, 11839–11844.
- Shoji, M., T.E.Golde, J.Ghiso, T.T.Cheung, S.Estus, L.M.Shaffer, X.D.Cai, D.M.McKay, R. Tintner, B.Frangione, and S.G.Younkin (1992) Production of the Alzheimer Amyloid β protein by normal proteolytic processing. *Science*, **258**, 126–129.
- Shivers, B.D., C.Hilbich, G.Multhaup, M.Salbaum, K.Beyreuther, and P.H.Seeburg (1988) Alzheimer's disease amyloidogenic glycoprotein: expression pattern in rat brain suggests a role in cell contact. *The EMBO Journal*, **7**, 1365–1370.

- Siman, R., S.Mistretta, J.T.Durkin, M.J.Savage, T.Loh, S.Trusko, and R.W.Scott (1993) Processing of the β -Amyloid Precursor (multiple proteases generate and degrade potentially amyloidogenic fragments). *The Journal of Biological Chemistry*, **268**, 16602–16609.
- Simons, M., E.Ikonen, P.J.Tienari, A.Cid-Arregui, U.Mönning, K.Beyreuther, and C.G.Dotti (1995) Intracellular routing of human Amyloid Protein Precursor: axonal delivery followed by transport to the dendrites. *Journal of Neuroscience Research*, **41**, 121–128.
- Simons, M., B.De Strooper, G.Multhaup, P.J.Tienari, C.G.Dotti, and K.Beyreuther (1996) Amyloidogenic processing of the human β Amyloid Precursor Protein in primary cultures of rat hippocampal neurons. *The Journal of Neuroscience*, **16**, 899–908.
- Sisodia, S.S. (1992) β -Amyloid Precursor Protein cleavage by a membrane-bound protease. *Proc. Natl. Acad. Sci.*, **89**, 6075–6079.
- Sisodia, S.S. E.H.Koo, P.N.Hoffman, G.Perry, and D.L.Price (1993) Identification and transport of full-length Amyloid Precursor Proteins in rat peripheral nervous system. *The Journal of Neuroscience*, **13**, 3136–3142.
- Stephens, D.J., and B.M.Austen (1996) Metabolites of the β -Amyloid Precursor Protein generated by β -secretase localise to the Trans-Golgi network, and late endosome in 293 cells. *Journal of Neuroscience Research*, **46**, 211–225.
- Suzuki, T., M.Oishi, D.R.Marshak, A.J.Czernik, A.C.Nairn, and P.Greengard (1994) Cell cycle-dependent regulation of the phosphorylation and metabolism of the Alzheimer Amyloid Precursor Protein. *The EMBO Journal*, **13**, 1114–1122.
- Tanzi, R.E., A.I.McClatchey, E.D.Lamperti, L.Villa-Komaroff, J.F.Gusella, and R.L.Neve (1988) Protease inhibitor domain encoded by an Amyloid Protein Precursor mRNA associated with Alzheimer's disease. *Nature*, **331**, 528–530.
- Thinakaran, G., D.B.Teplov, R.Siman, B.Greenberg, and S.S.Sisodia (1996) Metabolism of the “Swedish” Amyloid Precursor Protein variant in Neuro2a (N2a) cells (evidence that cleavage at the “ β -secretase” site occurs in the Golgi apparatus. *The Journal of Biological Chemistry*, **271**, 9390–9397.
- Tienari, P.J., B.De Strooper, E.Ikonen, M.Simons, A.Weidemann, C.Czech, T.Hartmann, N. Ida, G.Multhaup, C.L.Masters, F.Van Leuven, K.Beyreuther, and C.G.Dotti (1996) The β -amyloid domain is essential for axonal sorting of Amyloid Precursor Protein. *The EMBO Journal*, **15**, 5218–5229.
- Tienari, P.J., N.Ida, E.Ikonen, M.Simons, A.Weidemann, G.Multhaup, C.L.Masters, C.G.Dotti, and K.Beyreuther (1997) Intracellular and secreted Alzheimer β -amyloid species are generated by distinct mechanisms in cultured hippocampal neurons. *Proc. Natl. Acad. Sci.*, **94**, 4125–4130.
- Tischer, E., and B.Cordell (1996) β -Amyloid Precursor Protein. Location of Transmembrane Domain and specificity of secretase cleavage. *The journal of Biological Chemistry*, **271**, 21914–21919.
- Traub, L.M., and S.Kornfeld (1997) The Trans-Golgi network: a late secretory sorting station. *Current opinion in Cell Biology*, **9**, 527–533.
- Turner, R.S., N.Suzuki, S.C.Chyung, S.G.Younkin, and V.M.-Y.Lee (1996) Amyloid β 40 and β 42 are generated intracellularly in cultured human neurons and their secretion increases with maturation. *The Journal of Biological Chemistry*, **271**, 8966–8970.
- Walter, J., A.Capell, J.Grünberg, B.Pesold, A.Schindzielorz, R.Prior, M.B.Podlisny, P.Fraser, P.St.George-Hyslop, D.J.Selkoe, and C.Haass (1996) The Alzheimer's Disease-associated Presenilins are differentially phosphorylated proteins located predominantly within the endoplasmic reticulum. *Molecular Medicine*, **2**, 673–691.

- Walter, J., A.Capell, E.Y.Hung, H.Langen, M.Schnölzer, G.Thinakaran, S.S.Sisodia, D.J.Selkoe, and C.Haass (1997) Ectodomain phosphorylation of β -Amyloid Precursor Protein at two distinct cellular locations. *The Journal of Biological Chemistry*, **272**, 1896–1903.
- Weidemann, A., G.König, D.Bunke, P.Fischer, J.M.Salbaum, C.L.Masters, and K.Beyreuther (1989) Identification, biogenesis and localization of precursors of Alzheimer's disease A4 Amyloid Protein. *Cell*, **57**, 115–126.
- Wertkin, A.M., R.S.Turner, S.J.Pleasure, T.E.Golde, S.G.Younkin, J.Q.Trojanowski, and V.M.Y.Lee (1993) Human neurons derived from a teratocarcinoma cell line express solely the 695-amino acid Amyloid Precursor Protein and produce intracellular β -amyloid or A4 peptides. *Proc. Natl. Acad. Sci.*, **90**, 9513–9517.
- Wild-Bode, C, T.Yamazaki, A.Capell, U.Leimer, H.Steiner, Y.Ihara, and C.Haass (1997) Intracellular generation and accumulation of Amyloid β -peptide terminating at amino acid 42. *The Journal of Biological Chemistry*, **272**, 16085–16088.
- Yamada, T., H.Sasaki, H.Furuya, T.Miyata, I.Goto, and Y.Sakaki (1987) Complementary DNA for the mouse homolog of the human Amyloid β Protein Precursor. *Biochemical and biophysical research communications*, **149**, 665–671.
- Yamatsuji, T., T.Matsui, T.Okamoto, K.Komatsuzaki, S.Takeda, H.Fukumoto, T.Iwatsubo, N. Suzuki, A.Asami-Odaka, S.Ireland, B.Kinane, U.Giambarella, and I.Nishimoto (1996) G Protein-mediated neuronal DNA fragmentation induced by familial Alzheimer's disease-associated mutants of APP. *Science*, **272**, 1349–1352.
- Yamazaki, T., D.J.Selkoe, and E.H.Koo (1995) Trafficking of cell surface β -Amyloid Precursor Protein: retrograde and transcytotic transport in cultured neurons. *The Journal of Cell Biology*, **129**, 431–442.
- Yamazaki, T., E.H.Koo, and D.J.Selkoe (1996) Trafficking of cell-surface Amyloid β -Protein Precursor (endocytosis, recycling and lysosomal targeting detected by immunolocalization). *Journal of Cell Science*, **109**, 999–1008.
- Yankner, B.A., L.K.Duffy, and D.A.Kirschner (1990) Neurotrophic and neurotoxic effects of Amyloid β Protein: reversal by Tachykinin neuropeptides. *Science*, **250**, 279–282.
- Yonagisawa, K., A.Odaka, N.Suzuki, and Y.Ihora (1995) GM1 ganglioside-bound amyloid β -protein (Ab): a possible form of preamyloid in Alzheimer's Disease. *Nature Medicine*, **1**, 1062–1066.
- Yoshikawa, K., T.Aizawa, and Y.Hayashi (1992) Degeneration *in vitro* of post-mitotic neurons overexpressing the Alzheimer's amyloid precursor protein precursor. *Nature*, **359**, 64–67.
- Zhao, J., L.Paganini, L.Mucke, M.Gordon, L.Refolo, M.Carman, S.Sinha, T.Oltersdorf, I. Lieberburg, and L.McConlogue (1996) β -Secretase processing of the β -Amyloid Precursor Protein in transgenic mice is efficient in neurons but inefficient in astrocytes. *The Journal of Biological Chemistry*, **271**, 31407–31411.
- Zhong, Z., J.Higaki, K.Murakami, Y.Wang, R.Catalano, D.Quon, and B.Cordell (1994) Secretion of β -Amyloid Precursor Protein involves multiple cleavage sites. *The Journal of Biological Chemistry*, **269**, 627–632.

8. THE ROLE OF AMYLOID β PEPTIDE TERMINATING AT AMINO ACID 42 IN EARLY ONSET ALZHEIMER'S DISEASE

STEVEN G. YOUNKIN

Mayo Clinic Jacksonville, Jacksonville, Florida 32224

AMYLOID β PROTEIN DEPOSITION IN ALZHEIMER'S DISEASE

In patients with Alzheimer's disease (AD), large numbers of senile plaques are found throughout the cerebral neocortex and hippocampus. These senile plaques, which are present in small numbers in the brains of aged mammals and normal elderly individuals, are observed in large numbers only in AD and thus are specific for this disorder. Classic neuritic senile plaques consist of a spherical cluster of altered neurites surrounding an amyloid core composed of 5–10 nm wide fibrils that can be visualized on light microscopy by staining with Congo Red or Thioflavin S (Terry, 1985). In these plaques, microglia are intimately associated with the amyloid cores, and there are surrounding astrocytes with processes that project through the altered neurites toward the amyloid core. In many cases of AD, amyloid fibrils are also found in the walls of cerebral blood vessels (Glenner, 1983).

The principal proteinaceous component of the amyloid deposited in AD is an ~ 4 kD peptide, referred to as the amyloid β protein ($A\beta$), that has been isolated both from plaque cores and meningeal vessels of AD brain (Glenner and Wong, 1984; Masters *et al.*, 1985; Kang *et al.*, 1987; Prelli *et al.*, 1988; Mori *et al.*, 1992). Immunocytochemical studies with antisera to $A\beta$ have established that $A\beta$ is deposited not only in the neuritic plaques described above but also in large numbers of diffuse plaques, which are poorly circumscribed, immunoreactive lesions that are distinguished from neuritic plaques in that they show minimal neuritic change or glial reaction (Masliah *et al.*, 1990; Yamaguchi *et al.*, 1988).

$A\beta$ is produced by a single copy gene on chromosome 21, where it is encoded as an internal peptide within a large precursor protein referred to as the amyloid β protein precursor (β APP). In each of the various $A\beta$ -containing β APP isoforms produced through alternative splicing of the β APP gene, the 42 amino acid $A\beta$ peptide begins 99 residues from the carboxyl terminus of the β APP and it extends from the extracellular/intraluminal region (~ 28 amino acids) into the single membrane spanning domain (~ 14 amino acids) of the β APP (Kang *et al.*, 1987; Dyrks *et al.*, 1988). In 1992, several groups showed that normal processing of the β APP results in secretion of 4 kD $A\beta$ (Shoji *et al.*, 1992, Haass *et al.*, 1992, Seubert *et al.*, 1992) that is readily detected in human cerebrospinal fluid, plasma and medium conditioned by cultured cells. This soluble, secreted $A\beta$ is primarily $A\beta_{1-40}$ ($\sim 90\%$), but minor

amounts of A β 1–42 (~5–10%) and other A β species are also secreted (Dovey *et al.*, 1993, Vigo-Pelfrey *et al.*, 1993, Suzuki *et al.*, 1994).

Several groups have analyzed the insoluble A β that remains after the AD brain is extracted with high concentration SDS (Mori *et al.*, 1992; Roher *et al.*, 1993b; Roher *et al.*, 1993a; Miller *et al.*, 1993; Cowing *et al.*, 1994). The results of these studies indicate that SDS-insoluble amyloid cores are composed primarily of A β species ending at A β 42 whereas vascular amyloid is a mixture of A β ending at A β 40 and A β 42. Iwatsubo *et al.* (1994) examined a series of AD brains immunocytochemically using monoclonal antibodies specific for A β ending at A β 42 (BC-05) or A β 40 (BA-27). This analysis showed that BC-05 stains plaques of all types, that both BC-05 and BA-27 stain vessels, and that BA-27 stains plaques poorly compared to BC-05, in many cases only faintly labeling occasional plaque cores. Gravina *et al.* (1995) confirmed the immunocytochemical results of Iwatsubo *et al.* (1994) and, in addition, analyzed a series of 27 AD brains biochemically. To be sure that no A β was lost during isolation, Gravina *et al.* (1995) directly extracted the brains with 70% formic acid and then assayed with sandwich ELISAs specific for A β 40 or A β 42. In nine of the 27 AD brains examined, all of which had minimal A β deposition in vessels, virtually all (96%) of the A β deposited was A β 42. In an additional 7 brains, A β 42 was by far the predominant form deposited. Substantial amounts of A β 40 were observed only in brains with prominent deposition of A β in vessels. In a second immunocytochemical study, Iwatsubo *et al.* (1995) examined brains of trisomy 21 patients, who invariably develop AD pathology if they live past the age of 40. In brains from young patients, A β 42 was stained in diffuse plaques before any A β 40 was apparent. In the neuritic and cored plaques that were present in older brains, A β 42 was invariably intensely stained. Some A β 40 staining was detected in the older brains, particularly in mature cored plaques, but never at the intensity observed for A β 42. Collectively, these and similar studies have established that A β 42, a minor secreted form of A β , is deposited early and selectively in the senile plaques that are invariably observed in the AD brain.

EFFECT OF MUTATIONS THAT CAUSE EARLY ONSET FAMILIAL AD

It is now well established that early onset familial AD (FAD) can be caused by mutations in the amyloid β protein precursor gene (*APP*) on chromosome 21 (Goate *et al.*, 1991; Mullan *et al.*, 1992; Chartier-Harlin *et al.*, 1991), the presenilin 1 gene (*PS1*) on chromosome 14 (Sherrington *et al.*, 1995), and the presenilin 2 gene (*PS2*) on chromosome 1 (Levy-Lahad *et al.*, 1995, Rogaev, 1995). If A β deposition is an essential early event in the pathologic process that causes AD, then each of the genetic changes known to cause AD must cause changes that foster A β deposition. Thus one good way to test the A β deposition hypothesis is to determine if all the FAD linked mutations do, in fact, cause changes that foster A β deposition. There are many ways that these mutations might cause A β deposition. Our laboratory and several others have examined the hypothesis that the FAD-linked mutations act by increasing the extracellular concentration of A β .

To evaluate the *APP* mutations known to cause FAD, we and others examined fibroblasts (Citron *et al.*, 1994) from subjects carrying these mutations or transfected cells expressing the FAD-linked β APP mutations (Citron, 1992; Cai *et al.*, 1993; Suzuki *et al.*, 1994). These studies established that the FAD-linked mutations on the amino (β APP_{670N/671L}) and carboxyl (β APP_{717L}, F, or G) sides of A β do, in fact, alter β APP processing in a way that fosters amyloid deposition either by coordinately increasing the

extracellular concentration of A β 1–40 and A β 1–42(43) (β APP_{670N/671L}) (Cai *et al.*, 1993; Citron, 1992; Suzuki *et al.*, 1994) or by selectively increasing the extracellular concentration of A β 1–42(43) (β APP₇₁₇ mutations) (Suzuki *et al.*, 1994), a peptide that forms insoluble amyloid fibrils more rapidly than A β 1–40 *in vitro* (Jarrett *et al.*, 1993).

To determine if the FAD-linked *APP* mutations increase the extracellular concentration of A β *in vivo* and to assess the *PS1* and *PS2* mutations, we performed blinded comparisons of plasma A β 1–40 and A β 1–42 from carriers and controls (Scheuner *et al.*, 1996). Two studies were performed, the first compared 12 carriers with 31 noncarriers from the Swedish APPK_{670N,M671L} kindred, and the second compared 9 subjects with *PS1*_{G209V}, *PS1*_{M146V}, *PS1*_{H163R}, or *PS1*_{E120D} mutations; 3 subjects with *PS2*_{N141I} mutations; and one subject with an APP_{V717I} mutation with 14 controls. In addition, we analyzed plasma A β in 71 elderly patients with sporadic AD and 75 controls well matched for age, sex, and ethnicity. Remarkably, A β 1–42(43) was significantly increased in essentially identical fashion in the plasma of subjects with each type of mutated gene known to cause early onset familial AD. In the 12 subjects with *PS1/2* mutations, there was an unequivocal selective increase in the mean concentration of A β 1–42(43). As expected from previous studies, there was a coordinate increase in A β 1–40 and A β 1–42(43) in subjects with APP_{K670N,M671L} mutations but a selective increase in A β 1–42(43) in the subject with an APP_{V717I} mutation. Plasma A β 42(43) was increased in all of the presymptomatic carriers that were examined and it was *not* increased in the vast majority of symptomatic sporadic AD subjects that we examined. Thus, the elevated A β 42(43) observed in subjects with FAD-linked mutations is not a secondary phenomenon of the AD state.

There are many mechanisms that could foster the cerebral A β deposition that is invariably observed in all forms of AD. It appears that the mutations that cause early onset FAD may all cause deposition through a global increase in A β 42 that is measurable in plasma. A similar mechanism may operate in a small subset of sporadic AD patients because we observed elevated plasma A β 42 in the range associated with FAD in ~12% of the sporadic AD patients that we analyzed but in only 3% of controls ($p < 0.03$). Our analysis of sporadic AD clearly showed, however, that in most of these patients A β 42 deposition is not caused by a global increase in extracellular A β 42(43) concentration that is evident in plasma when disease is clinically apparent. In these sporadic AD cases, cerebral deposition of A β 42(43) must either be caused by a preexisting global increase in soluble, extracellular A β 42 that declines as A β is deposited (causing plasma A β 42 to fall into the normal range when symptoms are present) or by other factors such as a *local* increase in the extracellular concentration of A β 42(43), alterations in A β binding proteins (e.g. ApoE) that increase the rate of deposition, or an impairment of mechanisms that normally remove deposited A β .

To confirm that the FAD-linked *PS1/2* mutations increase A β 42, we examined the A β secreted by skin fibroblasts (Scheuner *et al.*, 1996). Fibroblast lines from 12 subjects with the *PS1*_{A246E} mutation, 10 subjects with the *PS1*_{L286V} mutation, 2 subjects with the *PS1*_{G209V} mutation, 2 subjects with the *PS1*_{M146V} mutation, and 3 subjects with the *PS2*_{N141I} mutation were compared with 38 fibroblast lines from controls. This comparison showed that A β 42 is also significantly elevated in media conditioned by fibroblasts from subjects with *PS1* or *PS2* mutations.

To establish that PS mutants increase brain A β , we analyzed the brains of transgenic mice expressing mutant (*PS1*M146L or *PS1*M146V) or wild type *PS1* that were developed by K. Duff (Duff *et al.*, 1996). This analysis showed unequivocally that overexpression of mutant *PS1* selectively

increases the endogenous A β 42 in mouse brain. Thus, it established that these mutations increase A β 42 not only in plasma and peripheral cells but also in brain, the target organ for AD pathology. In addition, this study showed that PS1 mutations act in a truly dominant fashion because the normal endogenous PS1 genes present in the mice that were expressing mutant PS1 did not prevent the increase in A β 42. In another study carried out collaboratively with Borchelt, Thinakaran, Sisodia and their colleagues at Johns Hopkins University (Borchelt *et al.*, 1996), we analyzed the brains of transgenic mice co-expressing β APP_{K670N,M671L} and either wild type PS1 or mutant PS1_{A246E}. In this experiment, the PS1_{A246E} mutation significantly increased the relative amount of brain A β 42, even though the amount of total brain A β was substantially increased by the overexpression of the APP transgene. In this same study, N2a cells co-expressing human β APP and either wild type or mutant PS1 (PS1_{M146L}, PS1_{A246E}, PS1 _{Δ E9}) were examined. Again, each of the mutations increased the relative amount of A β 42 found in the medium. In their study of transfected cells and transgenic mice expressing wild type or mutant presenilins, similar results were obtained by Citron *et al.* (1997).

Collectively, these studies of plasma, fibroblasts, transfected cells and transgenic mice show that a fundamental, generalized effect of the FAD-linked APP, PS1 and PS2 mutations is to increase the extracellular concentration of A β 42(43). The plasma data are particularly important because they establish that these mutations increase extracellular A β 42(43) *in vivo*. This effect of the FAD-linked mutations is likely to be directly related to the pathogenesis of AD because A β 42(43) is deposited early and selectively in the senile plaques that are an invariant feature of all forms of AD. Thus our results provide strong evidence that the FAD-linked mutations all cause AD by increasing the extracellular concentration of A β 42(43), thereby fostering A β deposition, and they support the hypothesis that cerebral A β deposition is an essential early event in the pathogenesis of all forms of AD. Other observations that provide additional support for a critical role for A β deposition in AD include (i) the finding that aggregated synthetic A β is toxic to cultured neurons *in vitro*, (ii) the observation that A β can trigger the classic complement cascade *in vitro*, and (iii) the finding that the A β deposited in neuritic plaques is intimately associated with proteins of the classic complement cascade and with reactive microglia likely to be releasing cytokines and reactive free radicals that could have neurotoxic effects.

Based on these observations, it is reasonable to propose that extracellular A β deposition initiates a pathologic cascade such as that shown in Figure 1 which involves the formation of senile plaques, neurofibrillary tangles, and ultimately neuron and/or synapse loss resulting in dementia. The available data are consistent with this model and strongly support it. Thus it is important to develop drugs that lower A β 42 or that prevent the aggregation and deposition of A β in other ways, as there is an excellent chance that such drugs will provide effective therapy for AD, if compounds can be identified that enter the brain in sufficient concentration to be effective without causing unacceptable toxicity.

Having said this, it should be noted that the close association between elevated extra-cellular A β 42 and the FAD-linked mutations does not necessarily imply a causal relationship between elevated A β 42 and the dementia that these mutations invariably produce. It is formally possible that the FAD-linked mutations both increase A β 42 and do something else (e.g. render neurons susceptible to age-related apoptosis) that causes dementia through a mechanism that is independent of the increase in A β 42. In this case, as shown in Figure 2, increased A β 42 would invariably be associated with the FAD-linked mutations, and it could cause aggregation, deposition, plaques, and perhaps even neurofibrillary tangle formation, but all of those changes would represent epiphenomena that are good markers of a different

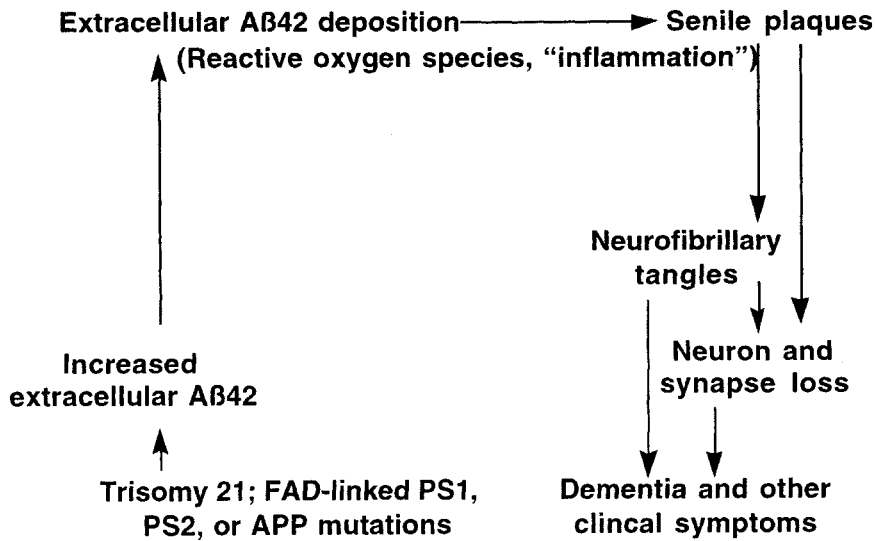


Figure 1.

process that is actually causing dementia. The well documented toxic effects of A β noted above make it highly unlikely that the elevated A β 42 produced by the various FAD-linked mutations is an innocent epiphenomenon. It is nonetheless worthwhile to continue testing the A β deposition hypothesis and to continue searching for other changes that are caused by the FAD-linked mutations. In this continuing investigation, it is important to recognize that, in some test systems, some of the FAD-linked mutations will undoubtedly have misleading, idiosyncratic effects that are unrelated to AD. Thus, in searching for effects that are related to AD, it is critically important to identify changes that are common to all of the various mutations and to establish that these common changes occur in multiple systems that are related as closely as possible to human brain.

The FAD-linked mutations may increase intracellular as well as secreted A β 42. It is far more difficult to detect intracellular A β than to detect secreted A β . It is currently unclear if this is because the amount of A β in cell lysates is small or because there are proteins in cells that make it technically difficult to detect intracellular A β . Wild-Bode *et al.* (1997) have reported that the β APP_{V717I} mutation increases intracellular as well as secreted A β 42, but the effects of other FAD-linked mutations is unknown. It is possible that the FAD-linked mutations cause only minor changes in intracellular A β that play no role in AD as illustrated in Figure 1. It is, however, important to investigate this issue further. The highest reported extracellular concentrations of A β , which have been measured in CSF, are well below the concentration necessary for spontaneous formation of amyloid fibrils *in vitro*. Thus the seeds which are critically important for fibril formation and A β deposition may form intracellularly in compartments where the local concentration of A β is high. If these seeds are rapidly externalized, then seed formation could be the only role of intracellular A β in AD. Alternatively, intracellular A β aggregates may cause substantial toxicity. If this is the case, then extracellular A β deposition could be a relatively innocent epiphenomenon that reflects the formation of intracellular A β aggregates which play a decisive role in the formation of senile plaques and neurofibrillary tangles and in the loss of

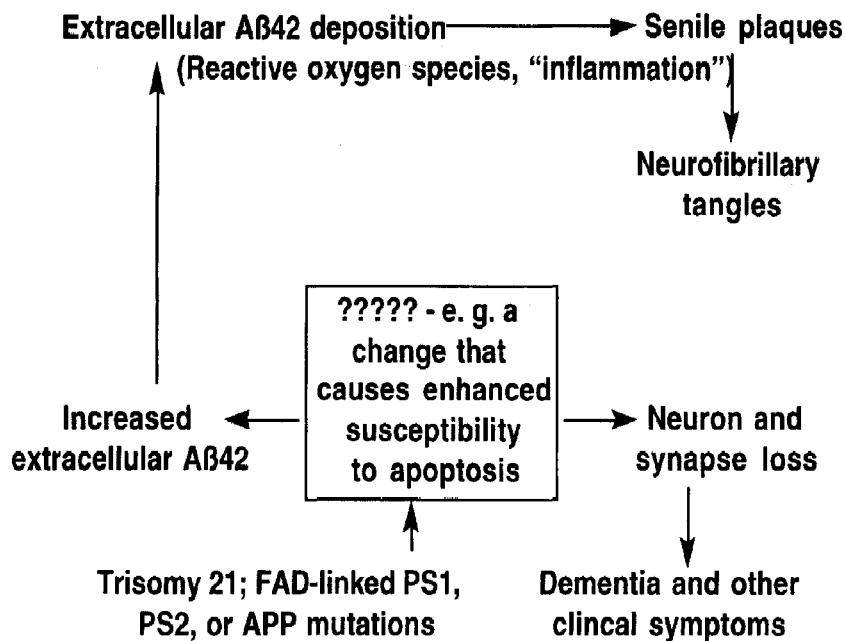


Figure 2.

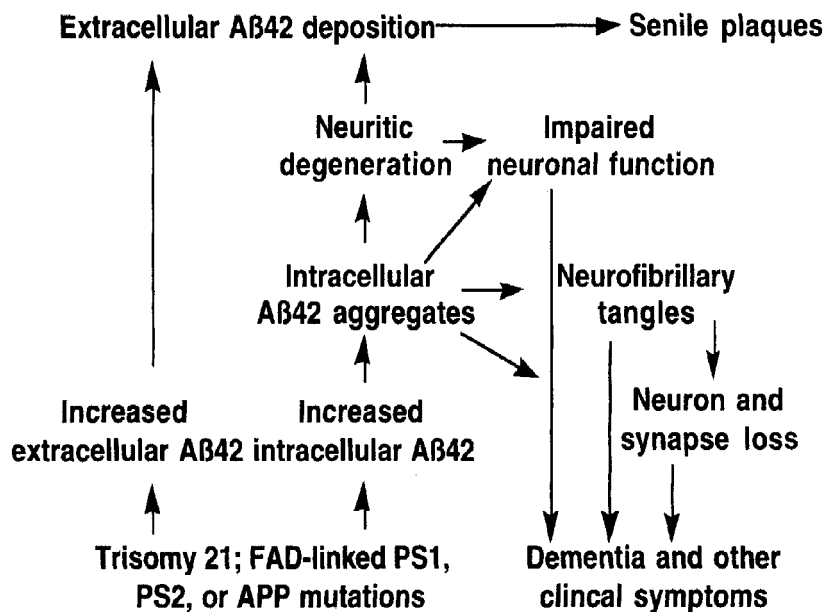


Figure 3.

neurons and synapses as shown in [Figure 3](#). It is even possible that intracellular A β aggregates contribute importantly to dementia by compromising neuronal function independently of any of the overt pathology observed in AD. If this occurs, then therapies that enable neurons to clear intracellular aggregates could have a profound beneficial effect even in cases of AD that are far advanced.

REFERENCES

- 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 1–42/1–40 ratio *in vitro* and *in vivo*. *Neuron*, **17**, 1005–13.
- Cai, X.D., Golde, T.E. and Younkin, S.G. (1993) Release of excess amyloid β protein from a mutant amyloid beta protein precursor [see comments]. *Science*, **259**, 514–6.
- Chartier-Harlin, M.C., Crawford, F., Houlden, H., Warren, A., Hughes, D., Fidani, L., Goate, A., Rossor, M., Roques, P., Hardy, J. *et al.* (1991) Early-onset Alzheimer's disease caused by mutations at codon 717 of the β -amyloid precursor protein gene. *Nature*, **353**, 844–6.
- Citron, M., Vigo-Pelfrey, C., Teplow, D.B., Miller, C., Schenk, D., Johnston, J., Winblad, B., Venizelos, N., Lannfelt, L., and Selkoe, D.J. (1994) Excessive production of amyloid *beta-protein* by peripheral cells of symptomatic and presymptomatic patients carrying the Swedish familial Alzheimer disease mutation. *Proc. Natl. Acad. Sci. USA*, **91**, 11993–7.
- Citron, M., Westaway, D., Xia, W., Carlson, G., Diehl, T., Levesque, G., Johnson-Wood, K., Lee, M., Seubert, P., Davis, A., Kholodenko, D., Motter, R., Sherrington, R., Perry, B., Yao, H., Strome, R., Lieberburg, I., Rommens, J., Kim, S., Schenk, D., Fraser, P., St George Hyslop, P., and Selkoe, D.J. (1997) Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid β -protein in both transfected cells and transgenic mice. *Nature Med.*, **3**, 67–72.
- Citron M., O.T., Haass, C., McConlogue, L., Hung, A.Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., and Selko, D.J. (1992) Mutation of the β -amyloid precursor protein in familial Alzheimer's disease increases β -protein production. *Nature*, **360**, 672–674.
- Dovey, H.F., Suomensaaari-Chrysler, S., Lieberburg, I., Sinha, S., and Keim, P.S. (1993) Cells with a familial Alzheimer's disease mutation produce authentic β -peptide. *Neuroreport*, **4**, 1039–42.
- Duff, K., Eckman, C., Zehr, C., Yu, X., Prada, C.M., Perez-tur, J., Hutton, M., Buee, L., Harigaya, Y., Yager, D., Morgan, D., Gordon, M.N., 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**, 710–3.
- Dyrks, T., Weidemann, A., Multhaup, G., Salbaum, J.M., Lemaire, H.G., Kang, J., Muller-Hill, B., Masters, C.L., and Beyreuther, K. (1988) Identification, transmembrane orientation and biogenesis of the amyloid A4 precursor of Alzheimer's disease. *EMBO J.*, **7**, 949–957.
- Glenner, G.G. (1983) In *Banbury Report 15: Biological aspects of Alzheimer's disease* (Ed, Katzman, R.) Cold Spring Harbor Laboratory, New York, pp. 137–144.
- 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**, 885–90.

- 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 [see comments]. *Nature*, **349**, 704–6.
- Gowing, E., Roher, A.E., Woods, A.S., Cotter, R.J., Chaney, M., Little, S.P., and Ball, M.J. (1994) Chemical characterization of A β 17–42 peptide, a component of diffuse amyloid deposits of Alzheimer disease. *J. Biol. Chem.*, **269**, 10987–90.
- Gravina, S.A., Ho, L., Eckman, C.B., Long, K.E., Otvos, L., Jr., Younkin, L.H., Suzuki, N., and Younkin, S.G. (1995) Amyloid beta protein (A β) in Alzheimer's disease brain. Biochemical and immunocytochemical analysis with antibodies specific for forms ending at A β 40 or A β 42(43). *J. Biol. Chem.*, **270**, 7013–6.
- Haass, C., Schlossmacher, M.G., Hung, A.Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B.L., Lieberburg, L., Koo, E.H., Schenk, D., Teplow, D.B., and Selkoe, D.J. (1992) Amyloid β peptide is produced by cultured cells during normal metabolism. *Nature*, **359**, 322–325.
- Iwatsubo, T., Mann, D.M., Odaka, A., Suzuki, N., and Ihara, Y. (1995) Amyloid β protein (A β) deposition: A β 42(43) precedes A β 40 in Down syndrome [see comments]. *Ann. Neurol.*, **37**, 294–9.
- Iwatsubo, T., Odaka, A., Suzuki, N., Mizusawa, H., Nukina, N., and Ihara, Y. (1994) Visualization of A β 42(43) and A β 40 in senile plaques with end-specific A β monoclonals: evidence that an initially deposited species is A beta 42 (43). *Neuron*, **13**, 45–53.
- Jarrett, J.T., Berger, E.P., and Lansbury, P.T., Jr. (1993) The carboxy terminus of the β amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry*, **32**, 4693–7.
- Kang, J., Lemaire, H.G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grzeschik, K.H., Multhaup, G., Beyreuther, K., and Muller-Hill, B. (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature*, **325**, 733–6.
- Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D.M., Oshima, J., Pettingell, W.H., Yu, C.E., Jondro, P.D., Schmidt, S.D., Wang, K., *et al.* (1995) Candidate gene for the chromosome 1 familial Alzheimer's disease locus [see comments]. *Science*, **269**, 973–7.
- Masliah, E., Terry, R.D., Mallory, M., Alford, M., and Hansen, L.A. (1990) Diffuse plaques do not accentuate synapse loss in Alzheimer's disease. *Am. J. Pathol.*, **137**, 1293–7.
- 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.
- Miller, D.L., Papayannopoulos, I.A., Styles, J., Bobin, S.A., Lin, Y.Y., Biemann, K., and Iqbal, K. (1993) Peptide compositions of the cerebrovascular and senile plaque core amyloid deposits of Alzheimer's disease. *Arch Biochem Biophys*, **301**, 41–52.
- Mori, H., Takio, K., Ogawara, M., and Selkoe, D.J. (1992) Mass spectrometry of purified amyloid β protein in Alzheimer's disease. *J. Biol. Chem.*, **267**, 17082–6.
- Mullan, M., Crawford, F., Axelman, K., Houlden, H., Lilius, L., Winblad, B., and Lannfelt, L. (1992) A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of β -amyloid. *Nat. Genet.*, **1**, 345–7.
- Prelli, F., Castano, E., Glenner, G.G., and Frangione, B. (1988) Differences between vascular and plaque core amyloid in Alzheimer's disease. *J. Neurochem.*, **51**, 648–51.
- Rogaev, E. (1995) Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. *Nature*, **376**, 775–778.

- Roher, A.E., Lowenson, J.D., Clarke, S., Wolkow, C., Wang, R., Cotter, R.J., Reardon, I.M., Zurcher-Neely, H.A., Heinrikson, R.L., Ball, M.J., *et al.* (1993a) Structural alterations in the peptide backbone of β -amyloid core protein may account for its deposition and stability in Alzheimer's disease. *J. Biol. Chem.*, **268**, 3072–83.
- Roher, A.E., Lowenson, J.D., Clarke, S., Woods, A.S., Cotter, R.J., Gowing, E., and Ball, M.J. (1993b) β -Amyloid-(1–42) is a major component of cerebrovascular amyloid deposits: implications for the pathology of Alzheimer disease. *Proc. Natl. Acad. Sci. USA*, **90**, 10836–40.
- Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T.D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-Lahad, E., Viitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wasco, W., Lannfelt, L., Selkoe, D., and Younkin, S. (1996) Secreted amyloid β -protein similar to that in the senile plaques of Alzheimer's disease is increased *in vivo* by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease [see comments]. *Nat. Med.*, **2**, 864–70.
- Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., Sinha, S., Schlossmacher, M., Whaley, J., Swindlehurst, C., McCormack, R., Wolfert, R., Selkoe, D., Lieberburg, I., and Schenk, D. (1992) Isolation and quantitation of soluble Alzheimer's β peptide from biological fluids. *Nature*, **359**, 325–327.
- 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 [see comments]. *Nature*, **375**, 754–60.
- Shoji, M., Golde, T.E., Ghiso, J., Cheung, T.T., Estus, S., Shaffer, L.M., Cai, X.D., McKay, D.M., Tintner, R., Frangione, B., *et al.* (1992) Production of the Alzheimer amyloid beta protein by normal proteolytic processing. *Science*, **258**, 126–9.
- Suzuki, N., Cheung, T.T., Cai, X.D., Odaka, A., Otvos, L., Jr., Eckman, C., Golde, T.E., and Younkin, S.G. (1994) An increased percentage of long amyloid beta protein secreted by familial amyloid β protein precursor (β APP717) mutants. *Science*, **264**, 1336–40.
- Terry, R.D. (1985) In *Textbook of Neuropathology* (Eds, Davis, R.L., and Robertson, D.M.) Williams & Wilkins, Baltimore, pp. 824–841.
- Vigo-Pelfrey, C., Lee, D., Keim, P., Lieberburg, I., and Schenk, D.B. (1993) Characterization of β -amyloid peptide from human cerebrospinal fluid. *J. Neurochem.*, **61**, 1965–8.
- Wild-Bode, C., Yamazaki, T., Capell, A., Leimer, U., Steiner, H., Ihara, Y., and Haass, C. (1997) Intracellular generation and accumulation of amyloid β -peptide terminating at amino acid 42. *J. Biol. Chem.*, **272**, 16085–16088.
- Yamaguchi, H., Hirai, S., Morimatsu, M., Shoji, M., and Harigaya, Y. (1988) Diffuse type of senile plaques in the brains of Alzheimer-type dementia. *Acta Neuropathol. (Berl.)*, **77**, 113–9.

9. THE BIOPHYSICS OF AMYLOID β -PROTEIN FIBRILLOGENESIS

DAVID B. TEPLow

*Center for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical
School 77 Avenue Louis Pasteur (HIM-756), Boston, MA 02115–5716 USA*

INTRODUCTION

The Societal Impact of Alzheimer's Disease

Alzheimer's disease (AD) is the fourth leading cause of death in the United States and the most common cause of dementia (Iqbal, 1991). There is currently no cure for the disease, nor are there effective treatments. As the disease inexorably progresses, tremendous physical, emotional, and economic burdens are placed on its sufferers and on their caregivers. In 1991, estimates of the direct yearly economic impact of AD in the U.S. exceeded \$20 billion (Ernst and Hay, 1994). Extrapolation of current statistics to the year 2040 suggests that the number of AD patients will quintuple (Iqbal, 1991). These statistics emphasize the importance of developing efficacious therapies for the disease.

The Pathobiology of Alzheimer's Disease

Disease treatment and/or prevention requires that the pathobiology of the disease be understood. Two invariant, and pathognomonic, histologic features of AD are extracellular amyloid plaques and intracellular neurofibrillary tangles (Selkoe, 1991). Plaques and tangles result from the aberrant deposition of two proteins, amyloid β -protein ($A\beta$) and tau (τ), respectively. $A\beta$ and τ are unrelated structurally and functionally, and have different patterns of deposition, yet each protein forms insoluble fibers which bind Congo Red and have cross- β pleated sheet secondary structure, characteristics exhibited by a broad variety of amyloids (Kirschner *et al.*, 1986). In this context, AD may be viewed as a “protein condensation” disorder.

Increasing immunohistochemical, biochemical, and genetic evidence supports a seminal role of $A\beta$ in the development of AD (Hardy, 1997a). $A\beta$ circulates in the plasma and cerebrospinal fluid primarily in two forms, 40 or 42 amino acids long ($A\beta_{40}$ or $A\beta_{42}$, respectively) (Scheuner *et al.*, 1996; Seubert *et al.*, 1992). $A\beta_{40}$ and $A\beta_{42}$ are produced through the action of multiple proteases, termed secretases, which cleave $A\beta$ from a large precursor molecule, the β -protein precursor (β PP) (Selkoe *et*

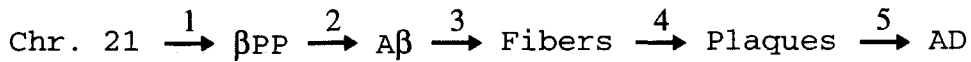


Figure 1. The role of A β in Alzheimer's disease. The amyloid β -protein (A β) is produced by proteolytic processing of the β -protein precursor (β PP), which is encoded by a single copy gene on human chromosome 21 (Chr. 21). For as yet unknown reasons, A β fibrillizes, leading to the formation of amyloid plaques which are nidi for the progressive destruction of the brain parenchyma.

al., 1995) (Figure 1, Step 2). In AD, A β polymerizes into fibers which self-associate to form the dense protein meshes characteristic of senile plaques (Figure 1, Steps 3 and 4). Plaques are actually complex structures which contain, in addition to A β , a variety of macromolecules, including apolipoprotein E, complement components, serum amyloid P, α_1 -antichymotrypsin, and proteoglycans (Dickson, 1997). Dystrophic neurites, characteristic of damaged neurons, can be seen within the plaque. Activated microglia are found around the periphery. Immunohistochemical studies suggest that A β_{42} is the earliest species to deposit in plaques, after which A β_{40} begins to accumulate (Lemere *et al.*, 1996). This observation is consistent with *in vitro* studies showing that A β_{42} forms fibers more rapidly than does A β_{40} (Jarrett *et al.*, 1993). Mutations associated with familial forms of AD (FAD) result in increased production of A β (Citron *et al.*, 1992), an elevated A β_{42} /A β_{40} ratio in the circulation (Scheuner *et al.*, 1996; Suzuki *et al.*, 1994), and increased plaque density (Strittmatter and Roses, 1996). Increased production of A β due to trisomy 21 (Figure 1, Step 1) is also thought to explain the invariant development of AD-like pathology in Down's syndrome patients living into their fifth decade (Mann and Esiri, 1989). A β deposition has, in fact, been observed in Down's syndrome patients as young as twelve years old (Lemere *et al.*, 1996). The above observations are consistent with the hypothesis that altered A β metabolism underlies the development of Alzheimer's disease (Hardy, 1997b).

Treatment Strategies

One strategy for preventing AD is inhibiting A β production. However, since A β is a normal product of cellular metabolism (Haass *et al.*, 1992; Seubert *et al.*, 1992), circulates in the plasma and cerebrospinal fluid, and is involved in organismal homeostasis in as yet undetermined ways, inhibiting A β production has the potential to produce unexpected and undesirable consequences. In contrast, A β fibrillogenesis is a pathologic event. Inhibiting this process could block the entire cascade of neurodegenerative events it initiates, without affecting normal physiology. When plaques and brain damage already exist, as is the case for most AD patients at the time of diagnosis, disaggregation of amyloid fibrils has the potential of halting the progression of the disease. For example, in patients with AL amyloid, which results from deposition of immunoglobulin L-chains, chemotherapy with the antimitotic agent iododoxorubicin resulted in disappearance of disseminated amyloid deposits and recovery of normal function in previously amyloid-laden organs (Gianni *et al.*, 1995). If strategies for amyloidogenesis inhibition and/or amyloid resorption are to be implemented successfully, detailed knowledge of amyloid metabolism must be acquired. It is logical, therefore, to begin by examining A β fibrillogenesis, the obligate first step in amyloid formation.

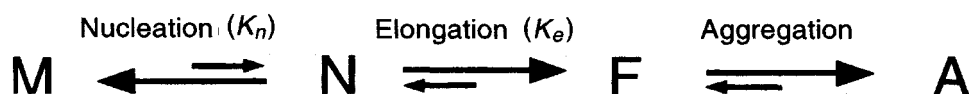


Figure 2. Simplified kinetic scheme of A β polymerization. A β polymerization is a nucleation-dependent polymerization process. Fibril nuclei (N) are formed through the thermodynamically unfavorable association of A β molecules (M). Fibrils (F) then elongate by addition of A β to fibril ends. Fibers and other endogenous macromolecules self-associate into supermolecular structures characteristic of Alzheimer amyloid (A). The kinetics of nucleation and elongation is characterized by the rate constants k_n and k_e , respectively.

Nucleation-Dependent Protein Polymerization

A β fibrillogenesis (Figure 2) is a nucleation-dependent polymerization process. The kinetics of this process is controlled by two key rate constants, those for nucleation (k_n) and elongation (k_e). Nucleation is a thermodynamically unfavorable reaction requiring the physical and temporal juxtaposition of numerous A β molecules of appropriate conformation. The nucleation rate thus displays an exponential dependence on A β concentration. Once nuclei form, fibers grow by subunit addition to the fiber ends. Elongation rate thus shows first order dependence on subunit concentration. Fiber-fiber interactions can produce a variety of higher-order structures resembling ropes, ribbons, and sheets (Fraser *et al.*, 1991). At sufficient concentration, gelation and fiber precipitation can occur. *A priori*, all of these processes have relevance to amyloid formation. In addition, non-A β components, such as metals, peptides, proteins, glycans, or lipids, may also modulate various aspects of these fibrillogenesis and post-fibrillogenesis processes. How then can we decide which stage(s) of this complex process to target in therapeutic studies? The answer to this question will emerge as each stage of A β fibrillogenesis is identified and corresponding rate constants are determined.

Establishing Useful Experimental Paradigms

Achieving a rigorous and complete understanding of A β fibrillogenesis requires the use of model systems yielding reproducible and controllable A β polymerization kinetics, from prenucleation phases through post-polymerization fiber-fiber assembly phases. Monitoring methods should be rapid, quantitative, non-invasive, and of high resolution, to allow realtime kinetic studies of polymerization stages without perturbing the underlying equilibria. Starting synthetic peptide preparations must be highly purified and free of polymeric material or particulate matter, both of which would confound analysis of fibril nucleation. End-products should display the morphologic, structural, and tinctorial characteristics of amyloid fibers. Two experimental systems will be discussed here. Each incorporates many of the features of the ideal system discussed above and each has yielded fundamental new information about A β fibrillogenesis.

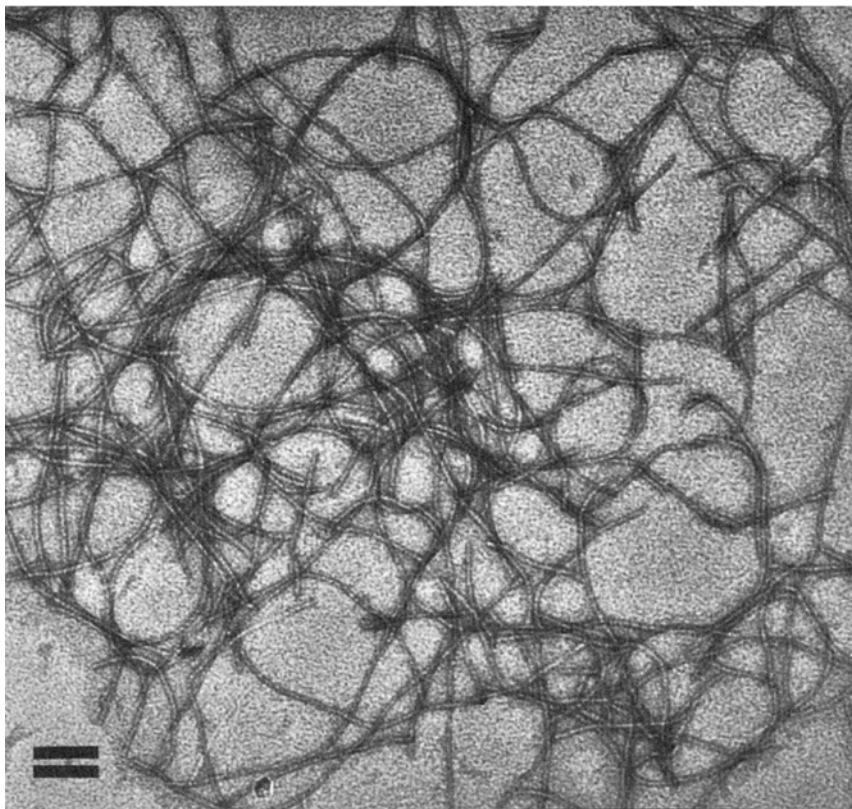


Figure 3. Morphology of A β polymers formed at low pH. Negative staining and electron microscopy revealed fibers of ~ 8 nm diameter (scale bar = 100 nm). These structures showed smooth margins, evidence of periodic internal structure, and little fiber-fiber interaction. These characteristics are similar to those of fibers isolated from AD plaque cores (Kirschner *et al.*, 1986) and produced at neutral pH *in vitro* (Harper *et al.*, 1997).

STUDIES OF THE NUCLEATION AND ELONGATION OF A β FIBRILS

A Model System for A β Fibril Formation

A β has the pathologically important, but experimentally vexing, potential for rapid self-association into fibrils and superfibrillar assemblies. This behavior has made the examination of fibril nucleation and elongation problematic. We found that highly reproducible and moderate rates of fibrillogenesis were achievable by dissolving and incubating synthetic A β_{40} in 0.1 N aqueous HCl at room temperature. Under these conditions, unbranched fibers ~ 8 nm in diameter were formed (Figure 3). These fibers were morphologically indistinguishable from those isolated from AD plaque cores (Kirschner *et al.*, 1986). Circular dichroism spectroscopy (CD) of the fibers showed them to possess $> 90\%$ β -strand/ β -turn character (Figure 4), consistent with results of CD, Fourier transform infrared spectroscopy, and

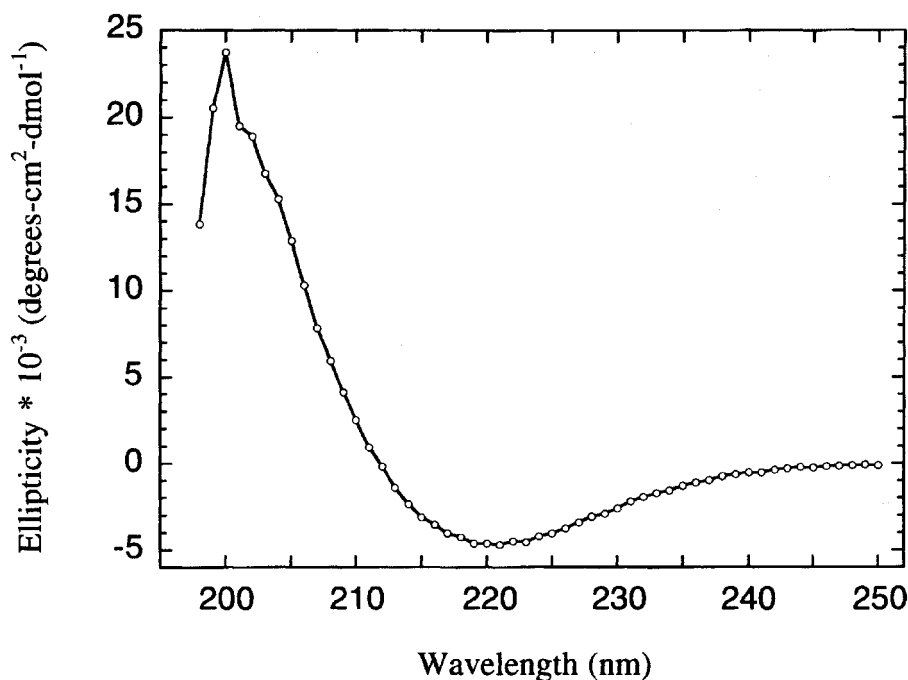


Figure 4. Circular dichroism spectroscopy of A β_{40} fibers formed at low pH. The minimum (220 nm) and maximum (200 nm) of the spectrum are characteristic of β -structure. Quantitative deconvolution of the spectrum was performed using CDANAL v. 1.0 (Perczel *et al.*, 1992).

fiber X-ray diffraction studies of fibers formed at neutral pH (Barrow *et al.*, 1992; Fraser *et al.*, 1992). In addition, the fibers bound Congo Red (Walsh, D.M. and Teplow, D.B., unpublished observations). The fibers formed at low pH thus exhibited the morphologic, structural, and tinctorial properties of amyloid fibers.

Monitoring A β Fibrillogenesis Using Quasielastic Light Scattering Spectroscopy (QLS)

QLS has long been recognized as a powerful means to study protein polymerization phenomena (Cohen and Benedek, 1975). QLS is an optical technique for the rapid, non-invasive, quantitative determination of the diffusion coefficients, D , of particles undergoing Brownian motion in solution (Pecora, 1985) (Figure 5). Diffusion coefficients can be converted into hydrodynamic radii, R_H , using the Stokes-Einstein relationship, $R_H = kTq^2 / 6\pi\eta D$; where k is the Boltzmann constant, T is temperature, q is the scattering vector (equivalent to $4\pi\eta \sin(\theta/2)/\lambda$, for scattering angle θ , and wavelength λ), and η is the solvent viscosity. R_H is the radius of a sphere with a diffusion coefficient equal to that of the scattering particle. For rod-shaped scatterers, such as A β fibrils, R_H is always smaller than the radius of the sphere described by free rotation of the fibril about its geometric center. In biochemical terms, R_H is analogous to a Stoke's radius in size exclusion chromatography. QLS was used to monitor the temporal change in R_H during the nucleation and elongation of A β fibrils.

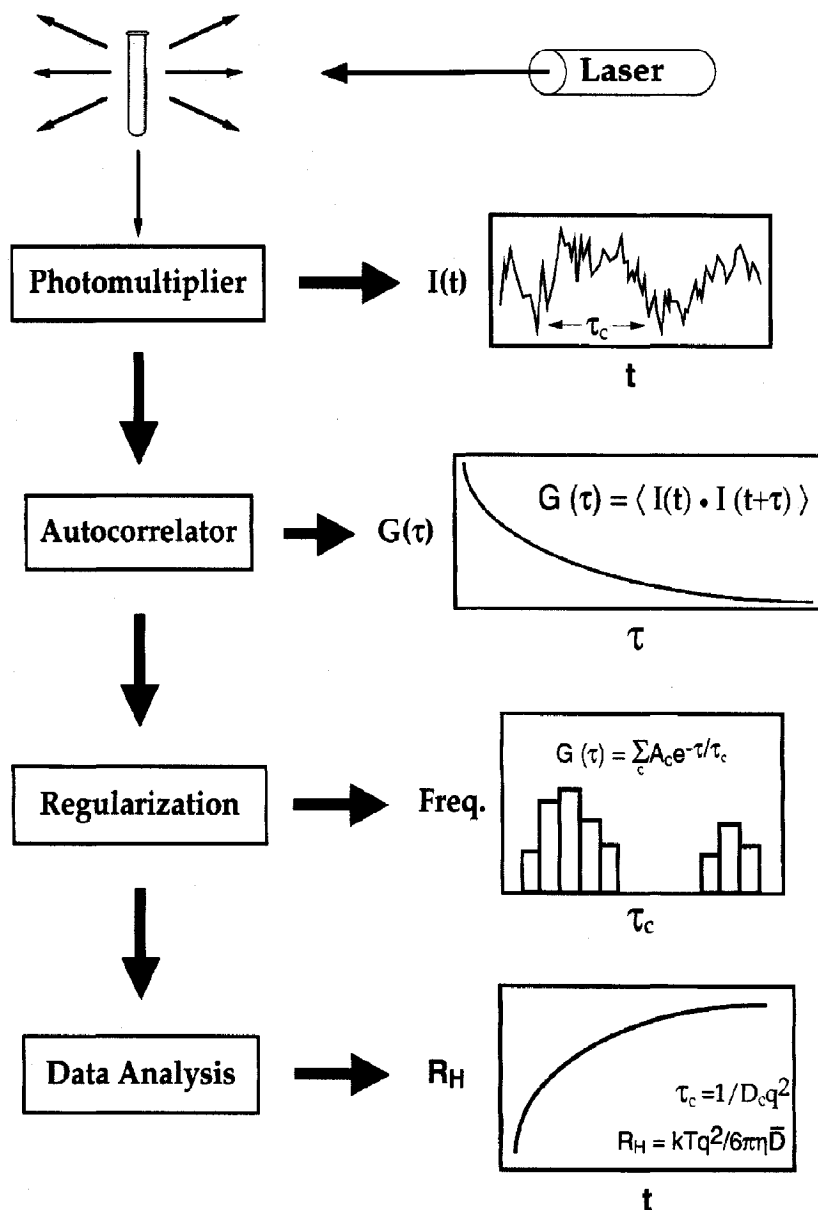


Figure 5. Quasielastic light scattering spectroscopy (QLS). In the QLS experiment, a laser is focused on a solution of particles undergoing Brownian motion. The temporal fluctuations in the intensity of light ($I(\tau)$) scattered by these particles are detected by a photomultiplier, which is connected to an autocorrelator. This instrument provides autocorrelation functions ($G(\tau)$) to a computer which subjects them to a regularization procedure. This produces a distribution of time constants (τ_c), from which the distribution of diffusion coefficients (D_c) is determined. Average hydrodynamic radii (R_H) may then be calculated using the Stokes-Einstein relationship. The temporal change in the R_H distribution is a sensitive measure of the nucleation and elongation of A β fibrils.

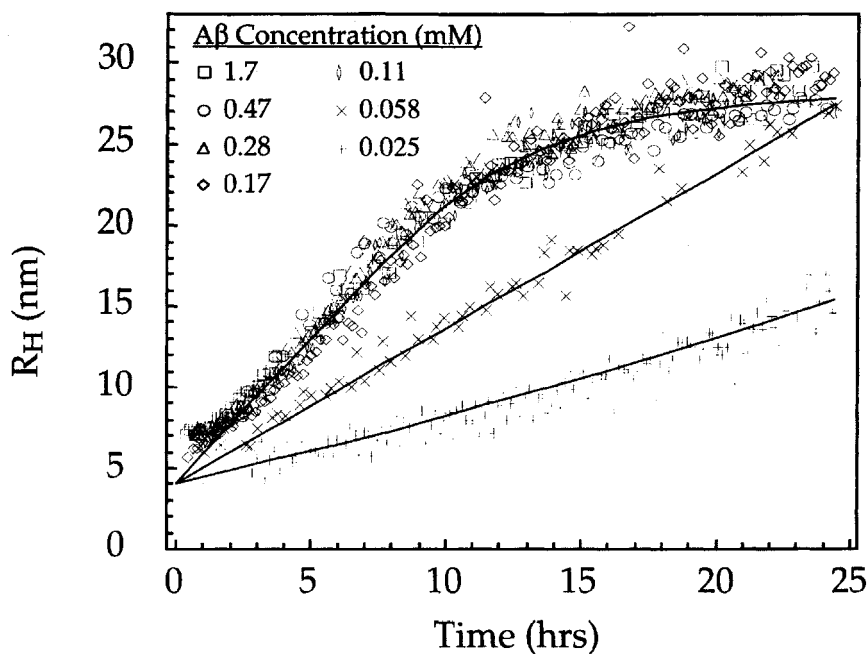


Figure 6. Concentration dependence of A β fibrillogenesis. QLS was used to monitor temporal changes in R_H during fibrillogenesis of A β_{40} . The initial A β concentration was varied over a range of approximately two decades. Solid lines are manual fits to the data. The extrapolated starting size of the A β oligomers was 4 nm. At higher concentrations, a 7 nm particle was observed transiently.

The Concentration Dependence of A β Fibrillogenesis

Concentration-dependence studies provide important information about the kinetic order of reactions. For this reason, we monitored the temporal changes in R_H in samples of A β_{40} dissolved at concentrations ranging from 25 μ M to 1.7 mM in 0.1 N HCl (Figure 6). Following the cessation of growth in R_H , aliquots were removed from each sample for electron microscopy and amino acid analysis. For all A β concentrations examined, the R_H increased over time, eventually reaching a constant level. The average intensity of the scattered light, a measure of the average molecular weight of the scattering particles, increased proportionately with R_H . These data suggested that A β was polymerizing into linear structures, and in fact, typical A β fibers were observed by electron microscopy (Lomakin *et al.*, 1996). The temporal change in R_H thus reflects a process in which fibrils grow linearly by the addition of A β subunits to fibril ends.

The kinetics of A β fibrillogenesis differed depending on whether the initial A β concentration, C_0 , did or did not exceed ~ 0.1 mM. Below this concentration, initial rates of fibril elongation were proportional to C_0 . As the pool of free A β was consumed by fibril growth, elongation slowed and eventually ceased. The extrapolated initial R_H for each experiment was ~ 4 nm. Particles of this size are likely to be fibril nuclei. The average fibril length observed when elongation ceased, L_f , displayed an inverse dependence on concentration (Figure 7). Interestingly, when $C_0 > 0.1$ mM, the fibrillogenesis kinetics was concentration independent. Over the full decade of concentrations examined (0.17–1.7

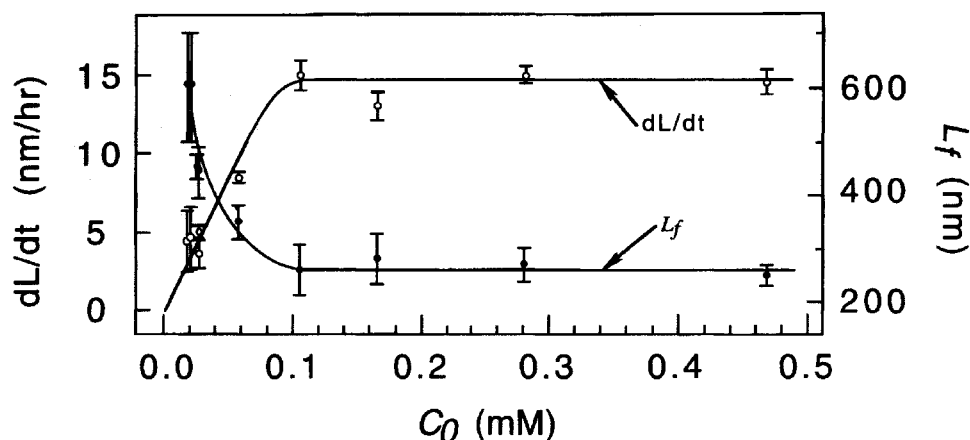


Figure 7. Concentration dependence of fibril elongation rate (dL/dt) and final fibril length (L_f). Fibril elongation rates were determined from data acquired during the linear phase of fibril growth. After fibril growth had ceased, final fibril lengths were calculated from R_H values using an interpolation appropriate for cylindrical scatterers 8 nm in diameter (de la Torre and Bloomfield, 1981).

mM), all data were superimposable (Figure 6). The initial R_H for each experiment, determined by extrapolation of the linear portion of each growth curve to $t=0$, was 4 nm, identical to that observed in the concentration domain $C_0 < 0.1$ mM. However, for $C_0 > 0.1$ mM, especially at higher concentrations, a lag phase was observed during which time the R_H remained constant at ~ 7 nm. The final average fibril length was independent of concentration in the domain $C_0 > 0.1$ mM (Figure 7). This L_f was significantly lower than the final average L_f values observed in experiments done at $C_0 < 0.1$ mM (Figure 7). It is informative to plot the initial fibril elongation rate, dL/dt

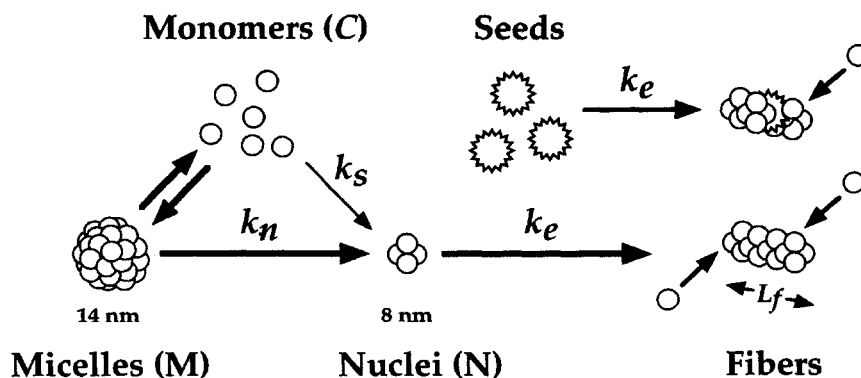


Figure 8. $A\beta$ fibrillogenesis at low pH. In the concentration domain $C_0 > c^*$, monomers are in rapid equilibrium with micelles (diameter = 14 nm), from which nuclei (diameter = 8 nm) emanate with a rate constant k_n . The rate of spontaneous nucleation, k_s , is extremely low, making nucleation from micelles the predominant nucleation process. Fibrils grow at the rate $k_e c^*$ by addition of $A\beta$ at the tips of the fibrils. For $C_0 < c^*$, micelles do not form, thus nucleation is mediated through both homogeneous (on $A\beta$) and heterogeneous (on seeds) mechanisms. At these lower $A\beta$ concentrations, nucleation on seeds predominates.

(determined during the initial phase of fibril growth when dL/dt shows a first-order dependence on time), versus initial A β concentration, C_0 (Figure 7). The direct and inverse relationships of dL/dt and L_f respectively, versus C_0 are apparent at concentrations below ~ 0.1 mM. Above 0.1 mM, however, no concentration dependence is seen. These data show that a phenomenon exhibiting a sharp concentration boundary is occurring at an A β concentration of ~ 0.1 mM. This concentration is referred to as the critical concentration, c^* .

A Model of A β Fibrillogenesis (Figure 8)

The above data are consistent with A β fibrils elongating by addition of A β precursors to fibril ends. The identity of these precursors is unknown. They could be monomers, dimers, or other oligomers, or particular conformers of one of these A β species. The rate of elongation for this type of polymerization process is first order with respect to precursor concentration. Thus, for $C_0 > c^*$, precursor concentration remains constant for approximately the first 10 hours of polymerization (Figure 6). Control of solute concentration over the decade of total A β concentrations examined is characteristic of condensed phases of matter, which form at concentrations above a solubility threshold and remain in rapid equilibrium with the soluble phase. What type of condensed phase might exist? The absence of detectable insoluble material is consistent with a microdispersion. The amphipathic nature of A β , and studies of the relationship of surface tension to A β concentration (Soreghan *et al.*, 1994), suggest formation of a microdispersion consisting of micelle-like particles. These micelles assemble spontaneously in the concentration domain $C_0 > c^*$, where $c^* \approx 0.1$ mM, giving rise to the 14 nm particles observed at high C_0 values.

Micellization has important effects on fibril nucleation and elongation. Relative to the concentration of free A β , micelles are volumes of high A β concentration. If micelles are modeled as spheres containing 25 A β molecules (Lomakin *et al.*, 1997), the intramicellar A β concentration would be ~ 30 mM, 300-fold higher than the concentration of free A β in the concentration domain $C_0 > c^*$. Because the fibril nucleation rate depends on the n -th power of A β concentration, where n is the number of A β molecules per nucleus, nucleation within micelles would occur orders of magnitude faster than in solution. This conclusion follows directly from calculation of the ratio of nucleation rates $(C_m/c^*)^n$ in the concentration domain $C_0 > c^*$, where C_m is the intramicellar A β concentration. Even for the most favorable nucleation process, trimerization ($n = 3$), this ratio is $> 2 \times 10^6$.

A β fibrillogenesis in the concentration domain $C_0 > c^*$ may be viewed as follows. Initially, and rapidly, A β assembles into micelles of $R_H = 7$ nm. Each micelle gives rise to a fibril nucleus at the rate $k_n [\text{sec}^{-1}]$. Once nuclei form, fibrils grow linearly through A β addition at fibril ends at the rate $k_e c^* [\text{sec}^{-1}]$. Although free A β is consumed in fibril elongation, its concentration, C , is held constant ($C \approx c^*$) due to the rapid equilibrium between micellar and free forms of A β . Thus the rate of fibril elongation remains constant ($k_e C = k_e c^*$). Micellar A β is eventually completely converted into free and fibrillar forms, at which point fibrillogenesis steadily consumes the free A β pool, lowering C and $k_e C$. This phase of the fibrillogenesis process manifests as a decline in dL/dT , eventually resulting in complete cessation of fiber growth.

In the concentration domain $C_0 < c^*$, micelles do not form. Nucleation must then occur either spontaneously or on preexistent nuclei formed from A β and/or contaminating non-A β material (seeds).

The rate constant for spontaneous (homogeneous) nucleation is small compared to that for nucleation within micelles ($k_s \ll k_n$). If fibrils form from seeds present in the peptide stock, the ratio C_0/N_0 , where N_0 = number concentration of preexistent nuclei, should be constant for all C_0 . This ratio also specifies final fibril length ($Lf = C_0/\lambda N_0$), where λ is linear density of A β in fibrils (1.6 monomers/nm). Thus Lf should also be constant. Experimentally, however, Lf increases as C_0 decreases, thus nucleation must involve cooperative interactions and not be mediated solely by preformed seeds. Once nuclei form, fibril elongation proceeds at the rate $k_e C$. As free A β is consumed through fibril growth, $k_e C$ decreases until it equals the rate of A β release from fibrils, at which point fibril growth is no longer apparent.

A full mathematical formulation of the above model has been published recently (Lomakin *et al.*, 1997). The formulation provides values for the key parameters of the model, k_n and k_e . It also enables determination of the time-dependent changes in monomer and micelle concentration, and in the concentration and size distribution of fibers, as fibrillogenesis proceeds. In the concentration domain $C > c^*$, the following values were obtained: $k_e = 90 \text{ M}^{-1} \times \text{sec}^{-1}$, $c^* = 0.1 \text{ mM}$, $k_n = 2.4 \times 10^{-6} \text{ sec}^{-1}$, $M_0 = 25$. Fibril nuclei thus form at a rate of approximately one per micelle every 5 days, while fibril growth from these nuclei proceeds at the rate of 0.5 monomers/min.

Assaying Fibrillogenesis Inhibitors

The ability to monitor and quantify the kinetics of prenucleation (micellization), nucleation, and fibril elongation suggested that the QLS system would be useful for assaying effects of potential inhibitors of fibrillogenesis. One such experiment is illustrated in Figure 9, where A β_{40} was dissolved and incubated at room temperature in 0.1 N HCl containing the non-ionic surfactant n-dodecylhexa-oxyethylene glycol monoether (C₁₂E₆). The final A β concentration was 0.11 mM and A β /C₁₂E₆ molar ratios of 2.5:1, 1:1, and 1:20 were employed. At the highest A β /C₁₂E₆ ratio, the initial elongation rate was identical to that previously observed in experiments with A β alone at $C > c^*$. Interestingly, the final fibril size, Lf , was significantly larger in the presence of C₁₂E₆ at the 2.5:1 ratio. Since $Lf \sim C_0/N_0$, these data are consistent with the interpretation that C₁₂E₆ is incorporated into micelles, which inhibits fibril nucleation, i.e. decreases N_0 . However, because fibrils elongated at equivalent rates both in the presence and absence of C₁₂E₆, the steady state concentration of free A β was unaffected by the surfactant. At C₁₂E₆/A β ratios of 1 and 20, both nucleation and elongation rates were diminished, while final fibril size was increased. As before, at constant total A β concentration, a decreased nucleation rate would produce an increase in the final average fibril length. The decrease in elongation rate is a direct result of lowering the effective concentration of A β capable of adding to fibril ends. These effects could result from formation of mixed micelles composed of A β and C₁₂E₆. In this model, c^* for the A β :C₁₂E₆ micelles would be lower than that of homogeneous A β micelles, explaining the decrease in elongation rate. It is also possible that C₁₂E₆ inhibits the β -strand transition required for fibril growth, an effect which could diminish both k_n and k_e .

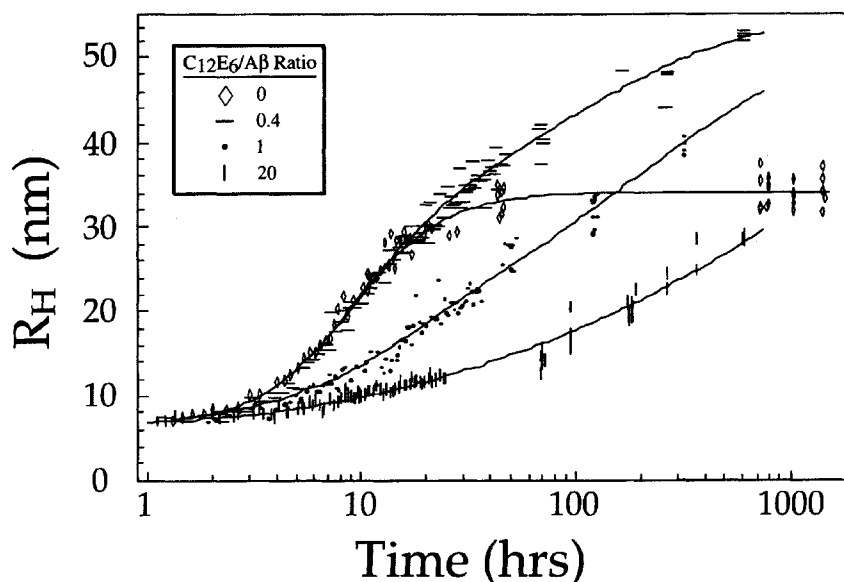


Figure 9. Effects of surfactant on A β fibrillogenesis. A β_{40} was dissolved at a final concentration of ~ 0.11 mM in 0.1 N HCl containing the non-ionic surfactant C₁₂E₆. Four different C₁₂E₆/A β ratios were examined, 0 (A β alone), 0.4, 1, and 20. The temporal change in A β R_H was then monitored by QLS

Correlation of Primary Structure with Fibrillogenesis Kinetics

It is axiomatic that the three-dimensional structure of a protein is determined by its amino acid sequence (Anfinsen, 1973). Single amino acid substitutions can effect remarkably profound changes in protein structure and activity, as for example, in sickle cell anemia. Missense mutations in the A β region of the β PP gene can result in similar effects. Hereditary Cerebral Hemorrhage with Amyloidosis-Dutch type (HCHWA-D) and congophilic amyloid angiopathy in a Flemish family are examples of amyloidoses caused by single amino acid substitutions in A β . In these disease, Glu₂₂→Gln (Dutch) and Ala₂₁→Gly (Flemish) substitutions result in the deposition of A β in vascular amyloid plaques, which leads eventually to cerebral hemorrhage and death. How these relatively minor changes in the primary structure of A β produce such dramatic alterations in peptide behavior is currently unknown.

In an effort to understand the molecular effects of the Dutch mutation, we synthesized A β_{40} peptide containing the Glu₂₂→Gln substitution and compared its fibrillogenesis kinetics with that of wild type A β . The peptide was dissolved at a concentration of 0.1 mM in 0.1 N HCl at room temperature, then the temporal change in R_H was measured by QLS. The average intensity of the scattered light was also monitored during the experiment. Electron microscopy was performed following the cessation of fibril growth.

A dramatic difference in behavior was exhibited by the Dutch peptide (Figure 10). In this molecule, a nominally minor side chain modification, changing a carboxylate to a carboxamide function, resulted in a 200-fold increase in initial elongation rate and an 8-fold increase in fibril length. In the

concentration domain $C > c^*$, final fibril size is given by the equation $L_f = [(k_{ec}^*/k_n)^{1/2}]/\lambda$ (Lomakin *et al.*, 1997). If the reasonable assumption is made that linear density is equal in both Dutch and wild type fibrils, then knowledge of L_f and k_{ec}^* allows solution of k_n for each peptide. Performing this calculation yields a ratio of nucleation rates, i.e. k_n (Dutch)/ k_n (wild type), of ~ 3 , demonstrating that the Glu₂₂→*Gln mutation accelerates *both* nucleation and elongation of fibrils.

Besides providing information on k_n and k_e , the QLS experiment also yields the temporal change in the average intensity, I , of the scattered light. By correlating I and R_H , information about scatterer structure can be obtained. For example, linear growth of fibrils is characterized by a direct proportionality between I and R_H . This phenomenon is readily apparent during the fibrillogenesis of wild type A β ₄₀ (Figure 11A), where the temporal change in I and R_H are closely correlated. In the case of the Dutch peptide, however, I increases disproportionately to R_H (Figure 11B). This behavior is characteristic of scattering particles which increase in molecular weight through *lateral* association. Thus the Glu₂₂→Gln substitution not only affects fibrillogenesis kinetics, but also promotes fiber-fiber associations which produce superfibrillar assemblies. The occurrence of these reactions in the cerebral vasculature of HCHWA-D patients might be one effector of the vascular pathology seen in the Dutch disease.

Summary

An experimental system was developed in which QLS was used to monitor the fibrillogenesis of synthetic A β under acidic conditions. This milieu produced moderate and reproducible

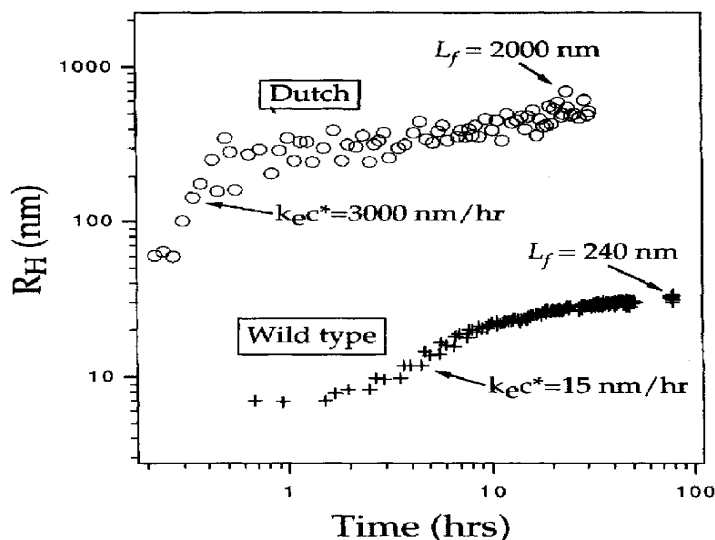


Figure 10. Effect of the Dutch amino acid substitution on the kinetics of A β fibrillogenesis. Wild type A β ₄₀, and A β ₄₀ containing the Glu₂₂→Gln (Dutch) mutation, were dissolved at concentrations of ~ 0.1 mM (c^*) in 0.1 N HCl. Fibril growth then was monitored by QLS. Growth rates were calculated from the temporal change in R_H during the linear phase of R_H increase. Final fibril lengths were calculated as in Figure 7. Note the log scale presentation.

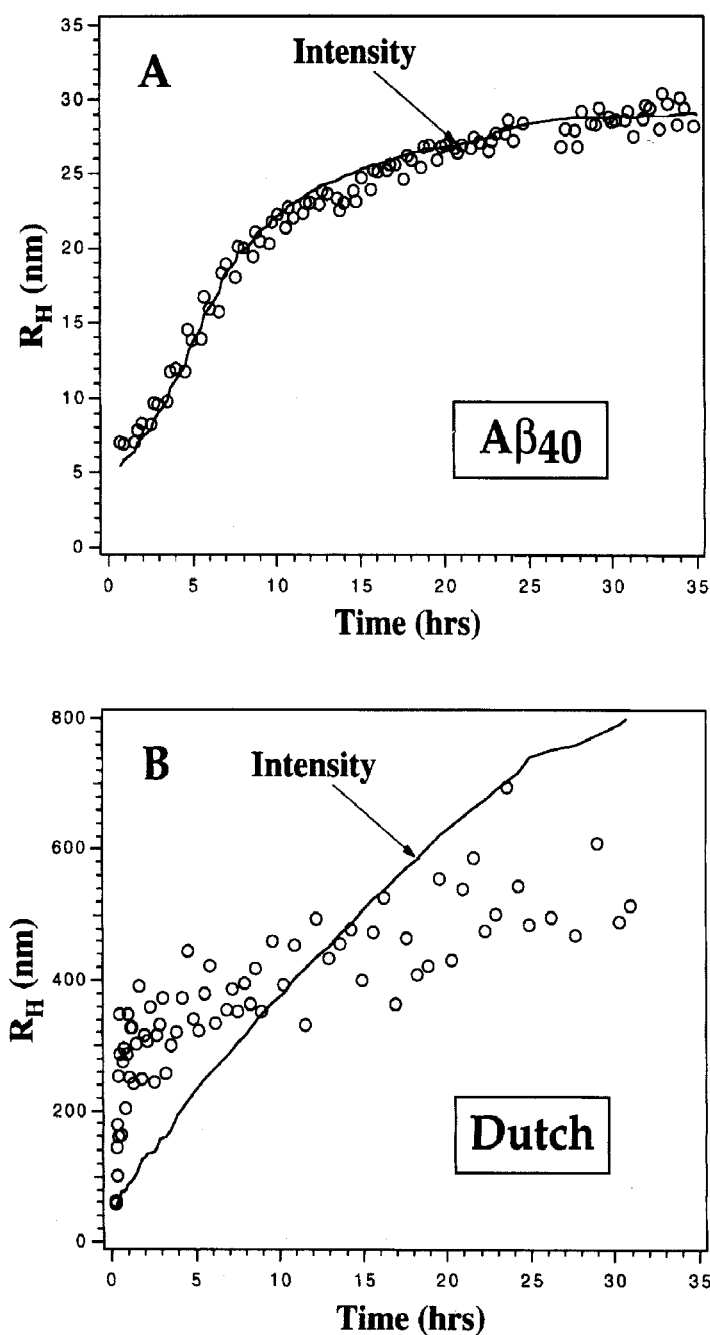


Figure 11. Effect of the Dutch amino acid substitution on fibril-fibril interactions. The growth of $A\beta_{40}$ fibrils was monitored by QLS. Scatter plots of the temporal change in R_H have been overlaid with normalized plots of the average scattered light intensity. (A) Colinearity of R_H and intensity plots from wild type $A\beta$ is consistent with a process of linear growth. (B) A disproportionate increase in intensity relative to R_H suggests that non-linear growth is induced by the Dutch mutation.

rates of A β fibrillogenesis, allowing the quantitative determination of A β polymer size throughout the fibrillogenesis process. Concentration-dependence studies showed that A β fibrillogenesis involved different mechanisms depending on whether the initial A β concentration exceeded c^* , the critical concentration for A β micellization. A conceptual model was developed which explained this phenomenon and which was used in the formulation of a set of equations for the temporal evolution of the fibril length distribution. This approach yielded the first quantitative determinations of the key kinetic parameters controlling fibrillogenesis, i.e. nucleation rate and elongation rate. In addition, it provided the means to determine fiber length and the diameters of nuclei and micelles. The value of these quantitative capabilities was illustrated in examinations of the mechanism of action of a potential fibrillogenesis inhibitor and the effects of amino acid substitutions within A β on the kinetics of fibrillogenesis. The methodological and theoretical approaches developed in these studies are now being extended in examinations of the temperature and pH-dependence of the fibrillogenesis process.

INTERMEDIATES IN A β FIBRILLOGENESIS

Introduction

Much is known about the macroscopic results of A β polymerization *in vivo*. Fibers grow, self-associate, and form amyloid deposits of extraordinary insolubility and protease resistance—deposits which are intimately associated with areas of damaged neuropil. Ideally, inhibiting fiber nucleation and the initial steps of fibril elongation would be the most effective approach to preventing the pathologic sequelae of amyloidosis. QLS studies have provided important information about the kinetics of fibril nucleation and growth, but more knowledge is needed about the structure and initial oligomerization reactions of nascent A β . For example, do A β monomers form fibril nuclei directly or do dimers or other oligomers associate to form these structures? What A β conformers add to the ends of growing fibrils? Are fibrillogenesis intermediates formed which might be targets for fibrillogenesis inhibitors? Answering these questions requires methodological approaches capable of resolving low abundance, low molecular weight A β species within complex mixtures of oligomers and polymers. Size exclusion chromatography (SEC) provides this capability.

SEC is a valuable chromatographic method for determining the Stokes radii of molecules in solution under non-denaturing, non-disaggregating conditions. By using soluble, globular proteins as standards, the proportionality between Stokes radius and molecular weight can be determined, enabling molecular weights of unknowns to be determined directly from chromatographic retention times. Molecular weight determination by SEC is based upon the assumption that the analyte is structurally and chromatographically isomorphous with the standards. If this is not the case, the determined molecular weight will be inaccurate. SEC is particularly powerful for the analysis of early multimerization events because of its capability for baseline resolution of monomers and dimers in the presence of oligomers and polymers. This capability complements an inherent difficulty in QLS, the inability to identify the scattered light intensity produced by low molecular weight species in the

presence of larger polymers. QLS, on the other hand, complements the inherent inability of SEC to accurately determine molecular weights of analytes displaying non-ideal chromatographic behavior, a potential problem with amyloid proteins (Irvine, 1997). I discuss here how SEC has been combined with QLS to analyze the earliest stages of A β fibrillogenesis.

Development of a Model System for A β Oligomerization

A β peptides were chemically synthesized, purified, and characterized as described (Lomakin *et al.*, 1996; Walsh *et al.*, 1997). To initiate oligomerization reactions, lyophilized material was dissolved in water, mixed with an equal volume of 2X buffer, and incubated for various time periods in sealed vials at room temperature. SEC was carried out on the supernates of samples centrifuged at 17,000 $\times$ g for 3 min. Centrifugation was done to remove large aggregates that might block solvent flow or function as nuclei for intracolumnar A β aggregation. The centrifugation does not remove nuclei, oligomers, or small polymers. Peaks were detected by UV absorbance at 254 nm. SEC was coupled to QLS by collection of fractions directly into QLS cuvettes. A summary of the SEC data is presented in Table 1.

Initial experiments using a Superdex 75 matrix to monitor polymerization of A β_{40} produced, at $t = 0$, a simple chromatogram containing a single peak with a M_r of 10,000. Although standardization of the column was excellent ($r^2 = 0.98$), the oligomerization state of the material in this peak could not be determined from the M_r alone due to the limitations of accuracy/precision inherent in the technique and because A β might have chromatographed non-ideally. Non-ideal behavior can occur through solute: column interaction and from deviation from globular structure. These issues were examined by determining if the M_r of A β changed in the presence of solvent additives affecting solute: column interactions and/or protein structure. Various mono- and polyhydroxylated compounds were studied, including ethylene glycol, sucrose, and 2-methyl-2,4-pentanediol (MPD). Modest variations in M_r were observed with sucrose and MPD, while increasing concentrations of ethylene glycol produced significant decreases of M_r . Because M_r did not remain constant, and column standardization was excellent ($r^2 \geq 0.96$ in every case), the data suggest that A β chromatographs non-ideally and/or the solvent additives alter its structure. Additional evidence for the non-ideal behavior of A β was obtained in experiments in which the same lot of A β was chromatographed on different matrices using the same buffer, or on the same matrix using different buffers. In each case, a single peak was observed but its M_r varied from 5,000–18,000. If the variation in M_r was due to multimerization and not non-ideal behavior, then dissociation should yield a consistent M_r . However, pretreatment of A β with dimethylsulfoxide, hexafluoroisopropanol, concentrated formic acid, or SDS-PAGE sample buffer had no effect on its M_r .

In an effort to improve the accuracy of molecular weights determined by SEC, QLS was employed to provide an absolute measure of the size of the protein molecules within each peak (Figure 12). To do so, A β was chromatographed as before on a Superdex 75 column eluted with Tris-HCl and the protein peak was collected directly into a cuvette and analyzed. The extrapolated M_r of this peak was 15,000. QLS showed that the peak contained particles with an average R_H of 1.8 ± 0.2 nm. By comparison, aprotinin (6,500 Da) had an average R_H of 1.6 ± 0.6 nm. The QLS data are thus consistent

with A β existing in Tris-HCl as a dimer. Recent experiments using fluorescence resonance energy transfer have also shown that A β_{40} forms stable dimers in Tris-HCl, pH 7.4 (Garzon-Rodriguez *et al.*, 1997). Formally, however, the possibility that A β exists as a monomer cannot be excluded based on the QLS data alone because changes in the tertiary structure and the degree of hydration of the A β particles alter the theoretical calculation of R_H . For simplicity, however, and with the preceding caveat noted, A β derived from the single, gel-included peak in the SEC experiments will be referred to as dimeric.

Discovery of the A β Protofibril

If fibrillogenesis involves formation of a post-nuclear intermediate(s), and if the intermediate forms at a rate exceeding that of its conversion into fibrils, then the intermediate will accumulate transiently. This possibility was examined by using SEC and QLS to monitor the temporal change in the distribution of A β_{40} oligomers in populations of molecules undergoing fibrillogenesis. A time-dependent decrease in dimer levels was observed under all conditions (Table 1) studied. In experiments using the Superdex 75/Tris-HCl system, this decrease in dimer concentration was accompanied by the appearance of a new peak in the void volume. The minimal size of the molecules in this peak can be estimated from the known exclusion limits of the Superdex column—100 kDa for globular proteins and 30 kDa for linear polymers, such as dextrans. To more accurately estimate the molecular weight of the molecules in the void peak, Superdex 200 and Superose 12 matrices, each with an exclusion limit of ~ 100 kDa (dextrans), were used. The A β multimers were excluded from both columns, showing that the molecular weights of the multimers exceeded 100 kDa, assuming their geometry was linear and that their exclusion resulted solely from their size. Analogous results were obtained in studies with A β_{42} (data not shown).

QLS was used as an independent method of characterizing the molecular features of the A β particles in the void peak. To do so, A β_{40} was incubated at a 2 mg/ml initial concentration for 48 h at room temperature in Tris-HCl, pH 7.4, then centrifuged and chromatographed as above. Column effluents were collected directly into QLS cuvettes and analyzed immediately. The void peak contained particles averaging ~ 20 nm in R_H (Figure 13). Particle sizes ranged from ~ 6 –70 nm. If the assumption is made that these particles are non-interacting rods of diameter 8 nm (Lomakin *et al.*, 1996), the R_H data could result from a population of A β oligomers ranging in length from ~ 20 –500 nm. The average particle size would be larger if the rods were flexible and smaller if rod-rod interactions occurred.

Table 1. Chromatographic Properties of A β (1–40)^a.

Column	Separation Range (kDa)	Elution Buffer ^b	A β M _r	Void Peak	Correlation Coefficient (r ²)
Superdex 75 (1 x 30 cm)	3–70	PBS	10,000	–	.98
		PBS/20% sucrose	10,500	–	.98
		PBS/20% MPD	9,000	–	.96
		PBS/20% Ethylene glycol	8,000	±	.98
		PBS/50% Ethylene glycol	6,000	±	.98
		0.1 M Tris-HCl, pH 7.4	15,000	+	.81
Superdex 200 (1 x 30 cm)	10–600	0.1 M Tris-HCl, pH 7.4	14,000	+	.77
Superose 12 (1 x 30 cm)	1–300	0.1 M Tris-HCl, pH 7.4	15,000	+	.79
		PBS	12,000	±	.92
TSK G2000sw (0.7 x 30 cm)	5–100	0.1% TFA	8,000	–	.85
		PBS	18,000	±	.58
50sf (1 x 28 cm)	1.5–30	0.1 M Tris-HCl, pH 7.4	9,000	–	.98
50sf (1 x 28 cm)		TBS	8,000	–	1.0
G50sf (1 x 47 cm)		TBSE	7,500	–	.98
G50sf (1 x 47 cm)		50 mM Ammonium acetate, pH 7.4	5,000	–	.98

^aA β (1–40) was chromatographed on five different size exclusion media, as described in Methods. For each study, the appropriate column was equilibrated with at least three column volumes of elution buffer and then calibrated with five molecular weight standards: avian ovalbumin (44,000); equine myoglobin (17,000); equine cytochrome C (12,384); bovine aprotinin (6,500); and vitamin B12 (1,350). Standard curves were constructed by regression analysis and used to determine the molecular weights of analytes. Each condition yielded one peak which decreased in size over 24 h. A small void peak appeared after 24 h incubation in some experiments. ^bMPD=2-methyl-2,4-pentanediol; PBS=phosphate-buffered saline; TBS=Tris-buffered saline; TBSE=0.02 M Tris-HCl, pH 7.4, containing 0.1 M NaCl and 50 μ M EDTA; TFA=trifluoroacetic acid.

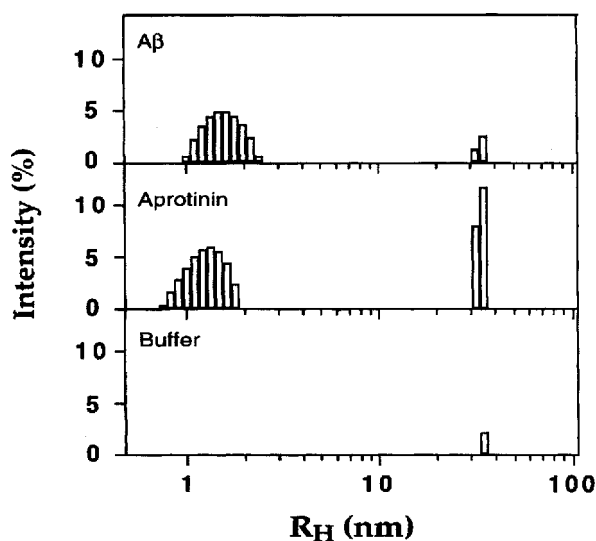


Figure 12. Fractionation and size determination of $A\beta$. $A\beta_{40}$ was dissolved in 0.1 M Tris-HCl, pH 7.4, and immediately fractionated on a Superdex 75 size exclusion matrix. The resulting single peak was collected directly into a QLS cuvette and analyzed. An average R_H of ~ 1.8 nm was observed. Aprotinin (6500 Da), chromatographed for comparison, yielded an R_H of ~ 1.6 nm. Scatterers of 30–40 nm in size are buffer related.

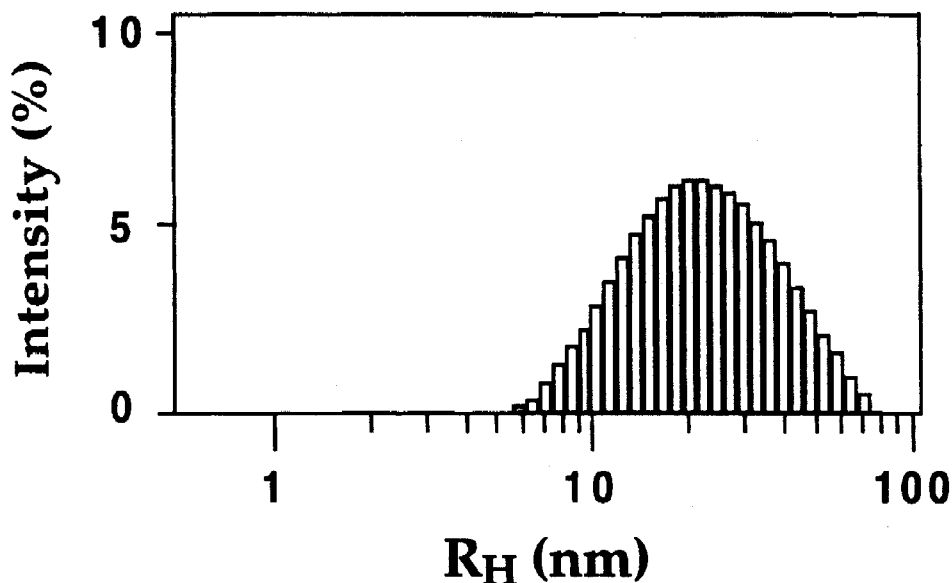


Figure 13. QLS analysis of gel-excluded $A\beta$ oligomers. $A\beta_{40}$, dissolved in 0.1 M Tris-HCl, pH 7.4, was incubated at room temperature for 48 h prior to size exclusion chromatography. The void volume peak then was collected and analyzed by QLS. A symmetrical distribution of particle sizes, centered at ~ 20 nm, was observed.

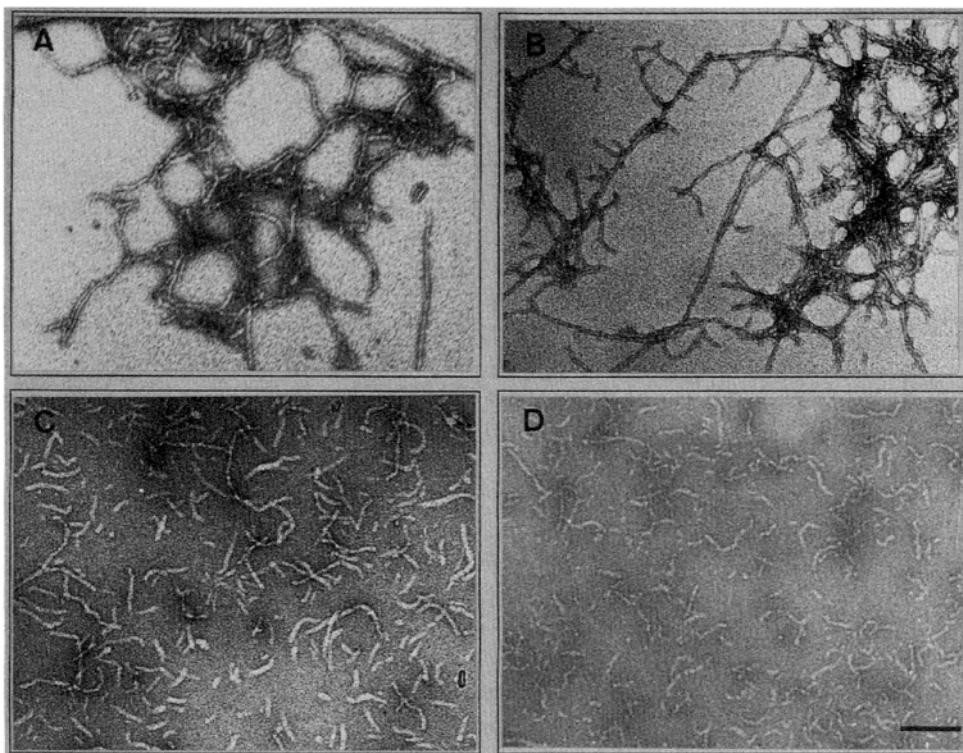


Figure 14. Morphology of A β polymers. A β_{40} and A β_{42} were incubated at room temperature for 6–48 hrs at an initial concentration of 2 mgs/ml in 0.1 M Tris-HCl, pH 7.4. After centrifugation at 17,000 $\times$ g for 3 min, the pellet was saved and the supernate was fractionated by SEC. The resulting void volume peaks and the original pellets were then negatively stained and examined by electron microscopy (scale bar=100 nm). (A) A β_{40} pellet; (B) A β_{42} pellet; (C) A β_{40} void fraction; and (D) A β_{42} void fraction.

Morphologic information about polymers of A β_{40} and A β_{42} was obtained through electron microscopic examination of SEC fractions. A β was incubated at an initial concentration of 2 mgs/ml in Tris-HCl, pH 7.4, for 6–48 h at room temperature before centrifugation at 17,000 $\times$ g for 3 min and chromatography. The resulting pellets and peak fractions were then spotted onto Formvar grids, fixed with glutaraldehyde, and negatively-stained with uranyl acetate (Figure 14). Pellets contained abundant enmeshed fibers 6–10 nm in diameter. These fibers resembled those isolated from amyloid plaques *in vivo* (Kirschner *et al.*, 1986). Unmeshed fibers of A β_{40} tended to be relatively straight, unbranched, and smooth (Figure 14A). Unmeshed fibers of A β_{42} were rather straight, often appearing as twisted pairs (Figure 14B). A prominent feature of the A β_{42} fibers was short fibrils which appeared to adhere to the fibers and to extend normal to the fiber axes. For both A β_{40} and A β_{42} , study of material isolated from the void peaks revealed numerous short, curved fibrils, 6–10 nm in diameter (Figures 14C and D). The lengths of these molecules generally varied from ~5–160 nm. We refer to these structures as “protofibrils,” to distinguish them from the amyloid-like fibers found in the pellets.

Kinetics of Protofibril Formation

To better understand the process of protofibril formation, A β peptides were dissolved in 0.1M Tris-HCl, pH 7.4, at a concentration of 1 mg/ml, and fibrillogenesis reactions allowed to proceed at room temperature for various time intervals. The distributions of polymer sizes then were determined by SEC using two different methods to detect A β within the resulting fractions, UV absorbance and inclusion of radioiodinated A β as a tracer. Initial experiments examined the behaviors of A β_{40} and A β_{42} .

As observed in the original SEC experiments, each peptide existed exclusively as a soluble dimer immediately upon dissolution (data not shown). By normalizing the peak heights of the dimer and protofibril fractions to that of the initial dimer peak, relative measures of dimer disappearance and protofibril formation were determined (Figure 15). Over 24 h, the level of A β_{40} dimers steadily decreased reaching ~80% of its initial value, while the level of A β_{42} dimers declined rapidly within the first hour, then more moderately, reaching ~20% of its initial value.

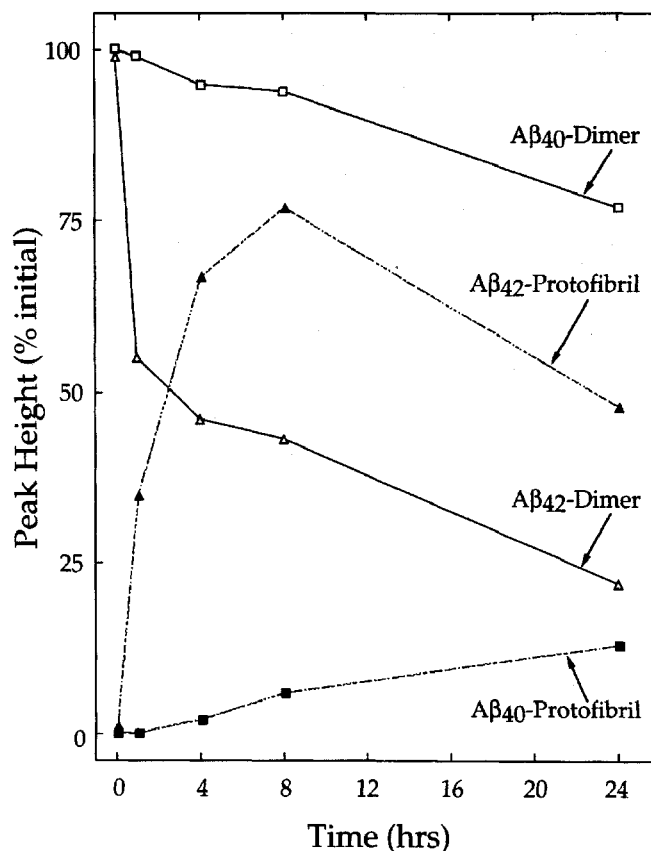


Figure 15. Kinetics of protofibril formation. A β_{40} and A β_{42} were dissolved at an initial concentration of 1 mg/ml in 0.1 M Tris-HCl, pH 7.4, incubated at room temperature for various times, then fractionated by SEC. Protofibril and dimer levels were determined by UV absorbance and normalized to the level of dimer at $t=0$.

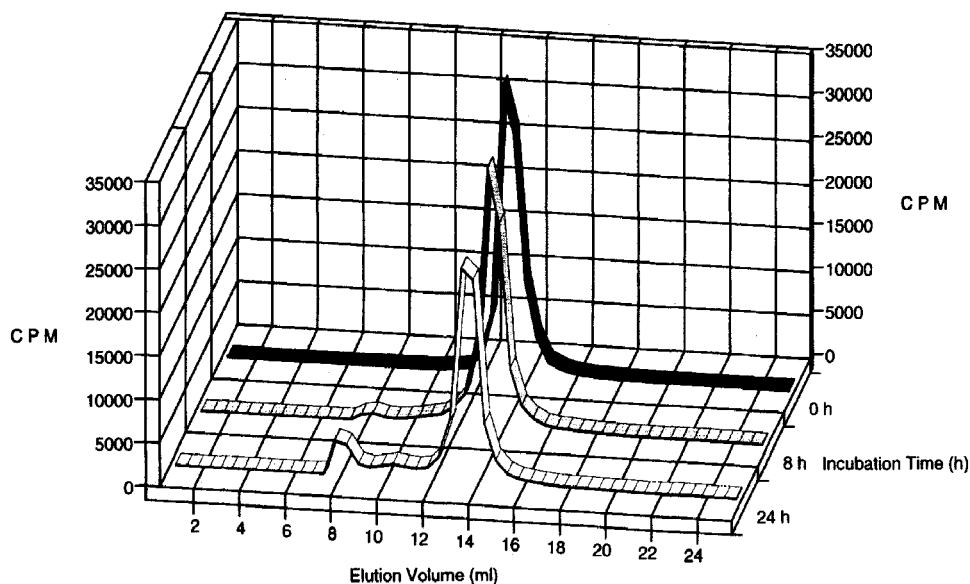


Figure 16. SEC analysis of fibrillogenesis of radiolabeled A β . A β_{40} was admixed with 100 pM radioiodinated A β_{40} then treated as in Figure 15. SEC fractions were analyzed by scintillation counting.

The temporal change in A β_{40} protofibril levels mirrored that of dimers. In the case of A β_{42} , protofibril levels rose quickly, reaching a peak after 8 h, then declined. This transitory appearance of protofibrils, occurring concomitantly with a decrease in dimer levels, is consistent with a fibrillogenesis process in which dimers are consumed in the formation of protofibrils, which are then, themselves, consumed to form fibers.

To determine whether other intermediates might have formed, but were present in amounts undetectable by UV absorbance, 100 pM radioiodinated A β_{40} was admixed with unlabeled peptide at 1 mg/ml (230 μ M) in 0.1 M Tris-HCl, pH 7.4. After intervals of incubation at room temperature, SEC was performed on a Superdex 75 system and radioactivity measured in each fraction by scintillation counting (Figure 16). At $t=0$, a single peak of radioactivity was observed at a retention time identical to that of the dimer peak as determined by UV absorbance. With increasing incubation time, a second peak appeared in the void volume of the column (Figure 16, ~ 7.4 ml). The temporal increase in radio activity in this fraction paralleled an observed increase in UV absorbance. No additional peaks were observed, although a time-dependent increase in the baseline between the void peak and the dimer peak was observed. This baseline change was also apparent by UV monitoring. The concordance of data from labeled and unlabeled A β fibrillogenesis experiments demonstrated that the labeled tracer peptide accurately reflected the polymerization behavior of the unlabeled bulk peptide in the fibrillogenesis reaction. The results demonstrate that as fibrillogenesis proceeds, the distribution of polymer sizes is characterized by a significant pool of dimeric A β molecules and protofibrils, and a low level continuum of oligomers. These data are consistent with a fibrillogenesis process in which A β

oligomerizes creating a continuum of short-lived multimeric intermediates which form protofibrils. Protofibrils are then consumed to produce fibers.

Effect of Amino Acid Substitutions in A β on Protofibril Formation

Earlier studies of A β fibrillogenesis at low pH showed that a small change in the primary structure of A β , e.g. the Glu₂₂→Gln mutation found in patients with HCHWA-D, could produce dramatic changes in the rates of fibril nucleation and elongation. To determine whether this substitution affected the kinetics of protofibril formation and utilization, fibrillogenesis reactions using A β ₄₀-Gln₂₂ were initiated in 0.1M Tris-HCl, pH 7.4, at room temperature, then monitored by SEC, as above. Two additional substitutions were also

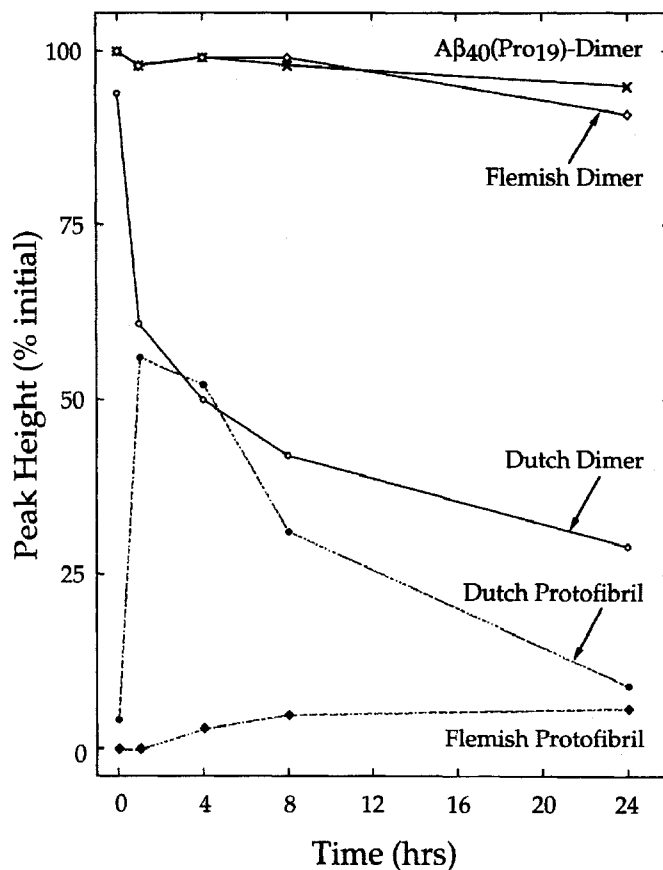


Figure 17. Effect of changes in A β primary structure on the kinetics of protofibril formation. A β ₄₀ containing the Dutch (Glu₂₂→Gln), the Flemish (Ala₂₁→Gly), or a Phe₁₉→Pro amino acid substitution were studied as in Figure 15.

studied, Ala₂₁→Gly, the Flemish mutation, and Phe₁₉→Pro, found to produce dramatic effects on β PP processing, presumably through structural alteration in the A β region of β PP (Haass *et al.*, 1994).

The Dutch peptide behaved similarly to A β ₄₂ (Figure 17; c.f. Figure 15). The level of the dimer peak fell ~40% within 1 h of incubation, then declined steadily to ~30% of its initial value. This decline was accompanied by a rapid rise in protofibril level, which peaked between 1 and 4 h, followed by a steady decline. Kinetically, the Glu₂₂ → Gln substitution produces an effect equivalent to that of adding the isoleucyl-alanine dipeptide to A β ₄₀. In contrast, the Flemish substitution decreased the rates of dimer utilization and protofibril formation (Figure 17) below those of wild type A β ₄₀. The Pro₁₉ substitution effectively prevented protofibril formation during the course of the experiment (24 h), a result consistent with the maintenance of initial levels of the Pro₁₉ dimer pool. Also consistent with this observation were results of prior QLS analyses of Pro₁₉ at low pH, which showed that the average R_H of the peptide remained essentially constant, at 1–2 nm, for days following dissolution (Teplow *et al.*, 1997). Studies of the secondary structure of the Pro₁₉ peptide suggest that the substitution prevents the random coil to β -strand conversion necessary for fibrillogenesis. For example, a 100 μ M solution of A β ₄₀-Pro₁₉ in 50 mM Tris-HCl, pH 7.4, remains in a random coil conformation for > 40 d at 37°C (Walsh, D.M. and Teplow, D.B. unpublished observation).

Taken together, these data show that structural alterations within or bounding the central hydrophobic region of A β (Leu₁₇-Glu₂₂), and at the A β COOH-terminus, exert significant control over the kinetics of protofibril formation and the conversion of protofibrils into fibers. Previous studies in which only late stages of the fibrillogenesis process were monitored have also suggested the importance of structural elements in the central and COOH-terminal regions of A β in controlling fibrillogenesis kinetics (Clements *et al.*, 1996; Jarrett *et al.*, 1993; Jarrett and Lansbury, 1993; Soto and Castano, 1996; Soto *et al.*, 1995). The structural basis of these effects is unknown, although it is clear that local changes in the structure of the peptide may affect both its intramolecular folding and intermolecular interactions.

Summary

Early phases of A β fibrillogenesis were studied using SEC to resolve oligomers. A β was found to chromatograph anomalously, making molecular weight estimations problematic. However, by combining QLS with SEC, a method was developed to determine oligomer size. This method was used to study the temporal evolution of the distribution of oligomer sizes, revealing the existence of a pool of low molecular weight A β molecules giving rise to protofibrillar intermediates. Kinetics experiments were consistent with a process in which protofibrils assembled into mature amyloid fibers. The combined SEC/QLS system provided the means to study factors controlling the kinetics of A β oligomerization and protofibril formation. In addition to its uses as an analytical tool, SEC of A β preparations may be useful as a preparative method for providing homogeneous, seed-free fractions of A β for studies of fibril nucleation. In addition, size-selected fractions of protofibrillar material could also be prepared for studies of A β seeding and fiber formation.

REFLECTIONS AND DIRECTIONS

A large body of genetic and biochemical evidence suggests that increased production of A β ₄₂, and its subsequent deposition in amyloid plaques, are seminal events in the pathogenesis of AD (Hardy,

1997b). Thus, a variety of approaches, many of which have been presented within this book, are being taken to understand the mechanisms of A β production and deposition. These mechanisms are complex, in no small part due to the interactions of a diversity of accessory molecules and factors, each of which may exert significant effects on the pathogenetic process (Figure 1). How, then, can we determine which steps in the process to target therapeutically? The answer is thoughtful simplification of the question.

The first step in the simplification process has been focusing on A β fibrillogenesis. Fibrillogenesis, in contrast to A β synthesis and secretion, is an abnormal process and one of the earliest steps in the pathogenetic cascade leading to AD. Therapeutic approaches targeting this process offer the hope of preventing AD or halting its progression at an early stage. Fibrillogenesis, however, is also a complex process. To be fully understood, each stage of fibrillogenesis must be characterized structurally and kinetically. Therefore, appropriate methods and theoretical tools must be developed. The use of QLS to monitor A β fibrillogenesis in a model fibrillogenesis milieu has provided the first estimations of A β fiber nucleation and elongation rates (Lomakin *et al.*, 1996), and of prenucleus and nucleus size. The QLS data also formed the basis for a mathematical formulation of the fibrillogenesis process which accurately represents the temporal evolution of the fiber length distribution (Lomakin *et al.*, 1997). Coupling QLS with SEC allowed standardization of the SEC systems, facilitating study of the earliest stages of A β oligomerization and characterization of a new fibrillogenesis intermediate, the protofibril (Walsh *et al.*, 1997).

We are now in a position to elucidate fully and quantitatively the effects of controlled perturbations in the fibrillogenesis system. Particularly important categories of effects are those resulting from changes in the primary structure of A β , the chemical nature of the fibrillogenesis milieu, and the constellation of non-A β macromolecules interacting with A β . Careful analysis of data derived from experiments in these areas will provide information valuable for the design of strategies to inhibit the fibrillogenesis process. The *in vitro* efficacy of potential inhibitors can then be assayed quantitatively using the approaches discussed here. Pilot studies of the effects of amino acid substitutions and the presence of surfactant on A β fibrillogenesis have been presented. Systematic studies of wild type A β_{40} and A β_{42} must now be done to define their fibrillogenesis behavior at neutral pH. These studies will serve as a baseline for subsequent experiments aimed at elucidating the key structural features in the A β molecule which control the kinetics of prenucleation, nucleation, protofibril formation, fiber elongation, and fiber-fiber association. Investigation of the effect of pH on these stages of fibrillogenesis could provide insight into differential behavior of A β in extracellular, cytosolic, and organellar compartments. The last and potentially hardest aspect of the fibrillogenesis process to understand is the effect(s) of the interaction of A β with the multitude of macromolecules it contacts, both intracellularly and extracellularly. The challenge inherent in these studies of A β fibrillogenesis will be to combine a rigorous understanding of the individual pieces of this process into a cohesive whole which accurately represents the complex and dynamic pathobiological events of Alzheimer's disease.

ACKNOWLEDGMENTS

I gratefully acknowledge the conceptual and experimental contributions of Drs. Aleksey Lomakin, Dominic M. Walsh, and Daniel A. Kirschner, and the technical assistance of Ms. Margaret Condrón and Ms. Karen Szumowski. Special recognition goes to my close collaborator, Dr. George B. Benedek, for his keen scientific insights and enthusiastic involvement in this work.

REFERENCES

- Anfinsen, C.B. (1973) Principles that govern the folding of protein chains. *Science*, **181**, 223–30.
- Barrow, C.J., Yasuda, A., Kenny, P.T.M., and Zagorski, M. (1992) Solution conformations and aggregational properties of synthetic amyloid β -peptides of Alzheimer's disease. *J. Mol. Biol.*, **225**, 1075–1093.
- Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A.Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., and Selkoe, D.J. (1992) Mutation of the β -amyloid precursor protein in familial Alzheimer's disease increases β -protein production. *Nature*, **360**, 672–674.
- Clements, A., Allsop, D., Walsh, D.M., and Williams, C.H. (1996) Aggregation and metal-binding properties of mutant forms of the amyloid A β peptide of Alzheimer's disease. *J. Neurochem.*, **66**, 740–747.
- Cohen, R.J., and Benedek, G.B. (1975) Immunoassay by light scattering spectroscopy. *Immunochem.*, **12**, 349–351.
- de la Torre, J.G., and Bloomfield, V.A. (1981) Hydrodynamic properties of complex, rigid, biological macromolecules: Theory and applications. *Quart. Rev. Biophys.*, **14**, 81–139.
- Dickson, D.W. (1997) The pathogenesis of senile plaques. *J. Neuropathol. Exp. Neurol.*, **56**, 321–339.
- Ernst, R.L., and Hay, J.W. (1994) The US economic and social costs of Alzheimer's disease revisited. *Am. J. Publ. Health*, **84**, 1261–1264.
- Fraser, P.E., Nguyen, J.T., Inouye, H., Surewicz, W.K., Selkoe, D.J., Podlisny, M.B., and Kirschner, D.A. (1992) Fibril formation by primate, rodent, and Dutch-hemorrhagic analogues of Alzheimer amyloid β -protein. *Biochem.*, **31**, 10716–10723.
- Fraser, P.E., Nguyen, J.T., Surewicz, W.K., and Kirschner, D.A. (1991) pH-dependent structural transitions of Alzheimer amyloid peptides. *Biophys. J.*, **60**, 1190–1201.
- Garzon-Rodriguez, W., Sepulveda-Becerra, M., Milton, S., and Glabe, C.G. (1997) Soluble amyloid A β -(1–40) exists as a stable dimer at low concentrations. *J. Biol. Chem.*, **272**, 21037–21044.
- Gianni, L., Bellotti, V., Gianni, A.M., and Merlini, G. (1995) New drug therapy of amyloidoses: resorption of AL-type deposits with 4'-iodo-4'-deoxydoxorubicin. *Blood*, **86**, 855–61.
- Haass, C., Hung, A.Y., Selkoe, D.J., and Teplow, D.B. (1994) Mutations associated with a locus for familial Alzheimer's disease result in alternative processing of amyloid β -protein precursor. *J. Biol. Chem.*, **269**, 17741–17748.
- Haass, C., Schlossmacher, M.G., Hung, A.Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B.L., Lieberburg, L., Koo, E.H., Schenk, D., et al. (1992) Amyloid β -peptide is produced by cultured cells during normal metabolism. *Nature*, **359**, 322–325.
- Hardy, J. (1997a) The Alzheimer family of diseases—Many etiologies, one pathogenesis. *Proc. Natl. Acad. Sci. USA*, **94**, 2095–2097.
- Hardy, J. (1997b) Amyloid, the presenilins and Alzheimer's disease. *Trends Neurosci.*, **20**, 154–159.

- Harper, J.D., Wong, S.S., Lieber, C.M., and Lansbury, P.T. (1997) Observation of metastable A β amyloid protofibrils by atomic force microscopy. *Chem. Biol.*, **4**, 119–125.
- Iqbal, K. (1991) Prevalence and neurobiology of Alzheimer's disease. In K.Iqbal, D.R.C.McLachlan, B.Winblad and H.M.Winsniewski, (eds.), *Alzheimer's Disease: Basic Mechanisms, Diagnosis and Therapeutic Strategies*, John Wiley and Sons, New York, 1–5.
- Irvine, G.B. (1997) Size-exclusion high-performance liquid chromatography of peptides. *Anal. Chim. Acta*, **352**, 387–397.
- Jarrett, J.T., Berger, E.P., and Lansbury, P.T., Jr (1993) The carboxy terminus of the β amyloid protein is critical for the seeding of amyloid formation: Implications for the pathogenesis of Alzheimer's disease. *Biochem.*, **32**, 4693–4697.
- Jarrett, J.T., and Lansbury, P.T., Jr (1993) Seeding “one-dimensional crystallization” of amyloid: A pathogenic mechanism in Alzheimer's disease and scrapie? *Cell*, **73**, 1055–1058.
- Kirschner, D.A., Abraham, C., and Selkoe, D.J. (1986) X-ray diffraction from intraneuronal paired helical filaments and extraneuronal amyloid fibers in Alzheimer's disease indicates cross β conformation. *Proc. Natl. Acad. Sci. USA*, **83**, 503–507.
- Lemere, C.A., Blusztajn, J.K., Yamaguchi, H., Wisniewski, T., Saido, T.C., and Selkoe, D.J. (1996) Sequence of deposition of heterogeneous amyloid β -peptides and APO E in Down syndrome-Implications for initial events in amyloid plaque formation. *Neurobiol. Dis.*, **3**, 16–32.
- Lomakin, A., Chung, D.S., Benedek, G.B., Kirschner, D.A., and Teplow, D.B. (1996) On the nucleation and growth of amyloid β -protein fibrils—Detection of nuclei and quantitation of rate constants. *Proc. Natl. Acad. Sci. USA*, **93**, 1125–1129.
- Lomakin, A., Teplow, D.B., Kirschner, D.A., and Benedek, G.B. (1997) Kinetic theory of fibrillogenesis of amyloid β -protein. *Proc. Natl. Acad. Sci. USA*, **94**, 7942–7947.
- Mann, D.M.A., and Esiri, M.M. (1989) The pattern of acquisition of plaques and tangles in the brains of patients under 50 years of age with Down's syndrome. *J. Neurol.*, **89**, 169–179.
- Pecora, R. (1985). *Dynamic Light Scattering: Applications of Photon Correlation Spectroscopy*, Plenum Press, New York, NY.
- Perczel, A., Park, K., and Fasman, G.D. (1992) Analysis of the circular dichroism spectrum of proteins using the Convex Constraint Algorithm: A practical guide. *Anal. Biochem.*, **203**, 83–93.
- Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T.D., Hardy, J., Hutton, M., *et al.* (1996) Secreted amyloid β -protein similar to that in the senile plaques of Alzheimer's disease is increased *in vivo* by the Presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nature Med.*, **2**, 864–870.
- Selkoe, D.J. (1991) The molecular pathology of Alzheimer's disease. *Neuron*, **6**, 487–498.
- Selkoe, D.J., Yamazaki, T., Citron, M., Podlisny, M.B., Koo, E.H., Teplow, D.B., and Haass, C. (1995) The role of APP processing and trafficking pathways in the formation of amyloid β -protein. *Ann. N.Y. Acad. Sci.*, **777**, 57–64.
- Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., Sinha, S., Schlossmacher, M.G., Whaley, J., *et al.* (1992) Isolation and quantitation of soluble Alzheimer's β -peptide from biological fluids. *Nature*, **359**, 325–327.
- Soreghan, B., Kosmoski, J., and Glabe, C. (1994) Surfactant properties of Alzheimer's A β peptides and the mechanism of amyloid aggregation. *J. Biol. Chem.*, **269**, 28551–28554.
- Soto, C., and Castano, E.M. (1996) The conformation of Alzheimer's beta peptide determines the rate of amyloid formation and its resistance to proteolysis. *Biochem. J.*, **314**, 701–707.

- Soto, C., Castano, E.M., Frangione, B., and Inestrosa, N.C. (1995) The α -helical to β -strand transition in the amino-terminal fragment of the amyloid β -peptide modulates amyloid formation. *J. Biol. Chem.*, **270**, 3063–3067.
- Strittmatter, W.J., and Roses, A.D. (1996) Apolipoprotein E and Alzheimer's disease. *Ann. Rev. Neurosci.*, **19**, 53–77.
- Suzuki, N., Cheung, T.T., Cai, X.-D., Odaka, A., Otvos, L., Jr, Eckman, C., Golde, T.E. and Younkin, S.G. (1994) An increased percentage of long amyloid β protein secreted by familial amyloid β protein precursor (β APP717) mutants. *Science*, **264**, 1336–1340.
- Teplow, D.B., Lomakin, A., Benedek, G.B., Kirschner, D.A., and Walsh, D.M. (1997) Effects of β -protein mutations on amyloid fibril nucleation and elongation. In K.Iqbal, B.Winblad, T. Nishimura, M.Takeda and H.M.Wisniewski, (eds.), *Alzheimer's Disease: Biology, Diagnosis and Therapeutics*, John Wiley & Sons Ltd., Chichester, England, 311–319.
- Walsh, D.M., Lomakin, A., Benedek, G.B., Condron, M.M., and Teplow, D.B. (1997) Amyloid β -protein fibrillogenesis—Detection of a protofibrillar intermediate. *J. Biol. Chem.*, **272**, 22364–22372.

THE PRESENILIN GENES

10.

THE MOLECULAR BIOLOGY OF PRESENILIN 1

GOPAL THINAKARAN¹ and SANGRAM S.SISODIA²

¹*Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA*

²*Department of Pharmacology and Physiological Sciences, University of Chicago, Chicago, IL 60637, USA*

SUMMARY

Mutations in two related genes, *PS1* and *PS2* account for the majority of early onset cases of familial Alzheimer's disease (FAD). *PS1* and *PS2* are polytopic membrane proteins which are predominantly localized in the endoplasmic reticulum. The precise mechanism(s) by which mutations in *PS* predispose individuals to FAD is not clear. However, analysis of plasma of affected individuals, conditioned medium from cells expressing FAD-linked *PS* variants, and transgenic mice expressing mutant *PS* reveal that mutant *PS* influences β -amyloid precursor protein (APP) processing in a manner leading to the production of elevated levels of highly toxic A β 42 peptides. The absence of marked changes in the levels of A β 42 in mice lacking one allele of *PS1* and functional rescue of the developmental abnormalities of *PS1*-deficient embryos by mutant *PS1* suggest that mutant *PS1* causes FAD not by loss of *PS1* activity during aging, but rather the gain of toxic property(ies).

PS1/PS2 EXPRESSION

PS1 and *PS2* mRNA are expressed in a variety of peripheral tissues and in the brain (Alzheimer's Disease Collaborative Group, 1995; Rogaev *et al.*, 1995; Sherrington *et al.*, 1995; Kovacs *et al.*, 1996; Cribbs *et al.*, 1996; Suzuki *et al.*, 1996; Sahara *et al.*, 1996; Boissière *et al.*, 1996; Page *et al.*, 1996; Lee *et al.*, 1996; Benkovic *et al.*, 1997). Although the structural conservation and relatively ubiquitous expression pattern of *PS1* and *PS2* mRNA suggest some degree of functional redundancy, differences exist in relative levels of expression suggesting that *PS1* and *PS2* may play different roles in tissue- or development-specific processes. Northern blotting has indicated that *PS2* mRNA are expressed at low levels relative to *PS1* mRNA (Rogaev *et al.*, 1995), but quantitative RT-PCR studies have revealed that *PS1* and *PS2* transcripts are expressed at significantly different levels among tissues and during brain development (Lee *et al.*, 1996). In the brains of adult mammals, both *PS1* and *PS2* transcripts are expressed in many neuronal populations (Kovacs *et al.*, 1996; Suzuki *et al.*, 1996; Lee *et al.*, 1996),

and the mRNA are also present in glial cells (Sherrington *et al.*, 1995; Rogaev *et al.*, 1995; Lee *et al.*, 1996). Notably, nerve cells known to be at risk in AD (i.e., neurons in hippocampal CA fields, in the medial and cortical amygdala, and in neocortex) express *PS* and *APP* transcripts at high levels, whereas neurons in regions less prone to AD-associated degeneration express *PS* and *APP* transcripts at more variable levels (Cribbs *et al.*, 1996; Page *et al.*, 1996; Boissière *et al.*, 1996; Lee *et al.*, 1996).

PS1 STRUCTURE AND LOCALIZATION

Using a variety of secondary structure algorithms, the 467 amino acid-long PS1 was predicted to contain between seven (Sherrington *et al.*, 1995) and nine transmembrane domains (Slunt *et al.*, 1995) and included a hydrophilic acidic "loop" region encompassing amino acids 262–407. The topology of PS1 was defined by two strategies: first, putative "transmembrane helices" were tested for their ability to export a protease-sensitive substrate across a lipid bilayer (Doan *et al.*, 1996); and, second, the plasma membrane of cultured cells that express human PS1 (HuPS1) were selectively permeabilized, and the accessibility of PS1 antibodies specific for the N-terminus, the loop, and the C-terminus to cognate epitopes was analyzed by indirect immunofluorescence microscopy. These studies established that the N-terminus, loop, and C-terminus of PS1 are oriented towards the cytoplasm (Doan *et al.*, 1996; De Strooper *et al.*, 1997). Similarly, the topology of the *C. elegans* presenilin homolog, termed "SEL-12," (see below) was determined using a series of SEL-12 β -galactosidase chimeras, an approach that relies on the observation that β -galactosidase is active in the cytoplasm of cells and is inactive in the lumen of membrane compartments. The deduced topology of SEL-12 indicated that the protein spans the membrane eight times, with N- and C-termini being exposed to the cytosol (Li and Greenwald, 1996). More recently, Lehmann *et al.* (1997) provided evidence for a six-transmembrane domain structure of PS1, wherein the C-terminal hydrophobic regions are associated with the cytosolic face but do not span the lipid bilayer.

Although the topology of PS2 has not been determined, it is highly likely that PS2 adopts a configuration not unlike PS1 and SEL-12. Significantly, the cytosolic domains of PS1 and PS2 are highly divergent (< 10% identity in the N-terminal 70 amino acids and between amino acids 305–375 in the loop domain), suggesting that these regions mediate cell- or PS-specific functions via differential interactions with proteins in the cytoplasm. Efforts aimed at the identification and characterization of molecules that interact with PS-specific cytosolic domains will be critical for the elucidation of the biological function(s) of this novel class of polytopic membrane proteins. In this regard, Zhou *et al.* (1997) utilized a yeast two-hybrid approach and identified δ -catenin as an interactor with the PS1 loop domain. Interestingly, δ -catenin is a member of a larger family of catenins related to a *Drosophila* protein termed "armadillo" involved in inductive signalling events during development. The influence of PS1 on δ -catenin function during development and in aging has not been determined.

Immunocytochemical analyses of a variety of cultured nonneuronal cells that express transiently expressed full-length PS1 and PS2 revealed that the proteins are localized to similar intracellular membranous compartments, including the endoplasmic reticulum, and to varying extent, the Golgi complex (Kovacs *et al.*, 1996; Cook *et al.*, 1996; Walter *et al.*, 1997; De Strooper *et al.*, 1997). In untransfected and stably transfected cells (in which PS1 fragments of ~27 and ~17 kD are preponderant), PS1 immunoreactivity is restricted to the endoplasmic reticulum (Walter *et al.*, 1997; Doan *et al.*, 1996;

Busciglio *et al.*, 1997). In cultured rat hippocampal neurons, PS1 staining was concentrated in the somatodendritic compartments but was also present at lower levels in axons (Busciglio *et al.*, 1997). Interestingly, the expression of epitope-tagged PS1 in a human neuronal cell line, NT2N, using a Semiki Forest virus delivery system-revealed that PS1 was localized primarily to the rough endoplasmic reticulum in cell bodies and dendrites but was excluded from axons and the cell surface (Cook *et al.*, 1996). However, recent studies also localized PS to the cell surface (Dewji and Singer, 1997), and to the nuclear membrane, kinetochores and the centrosomes (Li *et al.*, 1997).

Light microscopic immunocytochemical studies of rodent (Moussaoui *et al.*, 1996; Lee *et al.*, 1996), primate (Lah *et al.*, 1997), and human brains (Busciglio *et al.*, 1997) using antibodies selective for the N- or C-terminal PS1 fragments revealed that PS1 was present in all brain regions, with the strongest labeling in neurons and the neuropil, including axons and dendrites; weaker immunoreactivity was present in glial cells. Subcellular fractionation of monkey cortex revealed PS1 enrichment in nonsynaptic vesicle membrane compartments (Lah *et al.*, 1997). Notably, electron microscopic immunocytochemistry using an antibody specific for the PS1 N-terminal fragment disclosed selective PS1 immunoreactivity on cytoplasmic surfaces of smooth membranous organelles in cell bodies of neurons, suggesting localization in the endoplasmic reticulum-Golgi intermediate compartment and less-prominent localization in coated transport vesicles (Lah *et al.*, 1997). In the neuropil, PS1 immunoreactivity was present in dendritic spines and occasional presynaptic structures. In dendritic spines, PS1-immunoreactive structures had the general appearance of smooth tubular membranes or vesicles that resembled dendritic compartments of the smooth endoplasmic reticulum; no staining was observed on presynaptic structures, including synaptic vesicles (Lah *et al.*, 1997).

ENDOPROTEOLYSIS

The posttranslational processing of PS1 has been investigated in cultured cells and *in vivo*. Biochemical studies indicate that PS1/PS2 are not substrates for sulfation, glycosylation, or acylation (Cook *et al.*, 1996; Walter *et al.*, 1997; De Strooper *et al.*, 1997). However, serine residues in the N-terminus of PS2 (Walter *et al.*, 1997) and serine residues in the cytosolic loop domain of PS1 (Seeger *et al.*, 1997; Walter *et al.*, 1997; De Strooper *et al.*, 1997) are *in vivo* substrates for phosphorylation. PS1 phosphorylation is enhanced in response to activation of either protein kinase C or cAMP-dependent protein kinase or to the inhibition of protein phosphatase 1 or 2A (Seeger *et al.*, 1997; Walter *et al.*, 1997). The physiological significance of PS phosphorylation is not understood.

Although PS1 is synthesized as an ~42- to 43-kD polypeptide, the preponderant PS1-related species that accumulate in cultured mammalian cells and in the brains and systemic tissues of rodents, PS1 transgenic mice expressing HuPS1, primates, and humans are ~27-to 28-kD N-terminal (NTF) and ~16- to 17-kD C-terminal (CTF) derivatives (Thinakaran *et al.*, 1996; Mercken *et al.*, 1996; Lee *et al.*, 1996; Hendriks *et al.*, 1997; Podlisny *et al.*, 1997; Walter *et al.*, 1997). Epitope mapping studies indicated that PS1 is cleaved within a region that encompasses amino acids 260–320, a domain in which > 50% of identified FAD-linked PS1 mutations occur (Thinakaran *et al.*, 1996). Recent studies suggest that cleavage may be heterogenous, occurring between amino acids 292 and 299 (Podlisny *et al.*, 1997). These results are consistent with the demonstration that the FAD-linked PS1 Δ E9 variant, which lacks amino acids 290–319, fails to be cleaved (Thinakaran *et al.*, 1996).

The accumulation of ~27 kD and ~17 kD HuPS1-specific NTF and CTF in the brains of transgenic mice that express HuPS1 is highly regulated and saturable (Thinakaran *et al.*, 1996; Lee *et al.*, 1997); levels of PS1 derivatives are disproportionate to levels of transgene-derived mRNA or full-length HuPS1. The stoichiometry of accumulated ~27 kD NTF and ~17 kD CTF is ~1:1 in nontransgenic and transgenic mouse brains; this ratio is independent of the level of transgene-derived HuPS1 mRNA expression (Thinakaran *et al.*, 1996). The mechanism(s) involved in regulating the levels of accumulated PS1 derivatives have not been fully established, but several important insights have emerged. For example, in transfected cells overexpressing PS1 or PS2, only a small fraction of newly synthesized full-length PS1 and PS2 are converted to fragments while the remaining full-length polypeptides are rapidly degraded (Ratovitski *et al.*, 1997; Podlisny *et al.*, 1997; Kim *et al.*, 1997a). Recent studies demonstrated that when expressed at high levels in cultured cells, PS2 is polyubiquitinated and degraded by a lactacystin-sensitive proteasome pathway (Kim *et al.*, 1997a). In contrast to PS2, PS1 does not appear to be polyubiquitinated (Thinakaran and Sisodia, unpublished observations).

The observation that PS1 or PS2 fragment accumulate to 1:1 stoichiometry suggested that the NTFs and CTFs may co-associate in higher order assemblies. Two lines of evidence support the idea that N- and C-terminal PS1 or PS2 derivatives are co-resident: in cultured mammalian cells, these fragments can be specifically cross-linked *in situ* using dithiobis(succinimidylpropionate) (DSP), a membrane-permeable sulfhydryl-cleavable crosslinking agent which cross-links primary amines of adjacent proteins; and, the two fragments can be co-immunoprecipitated from mild detergent lysates of cultured cells or mammalian brain (Thinakaran *et al.*, 1998). Moreover, gel filtration chromatography and sucrose density gradient fractionation of nonionic detergent lysates from cultured cells reveal that PS1 N- and C-terminal fragments appear to remain associated in larger complexes of ~100 kD (Seeger *et al.*, 1997). It is not presently clear whether the co-isolated fragments are in homomeric or heteromeric assemblies.

A conserved feature of the topology models for the PS homologues is that the site for endoproteolytic cleavage is located in the cytosolic compartment. At present, neither the identity of the protease nor the physiological significance of PS1 proteolysis is known. Notably, FAD-linked PS1 missense mutations do not seem to affect endoproteolysis (see below). However, in view of the demonstration of a paucity of full-length PS1 and highly regulated accumulation of processed derivatives *in vivo*, it is highly likely that PS1 fragments are the "functional units" (Thinakaran *et al.*, 1996). Moreover, HuPS2 (Citron *et al.*, 1997; Kim *et al.*, 1997a; Tomita *et al.*, 1997) and SEL-12- β -galactosidase chimeras (Li and Greenwald, 1996) are also subject to endoproteolytic cleavage. This observation indicates that endoproteolysis of the protein is a highly conserved process and, arguably, a processing event that regulates the accumulation of fragments. Remarkably, in mouse N2a cell lines and in brains of transgenic mice expressing HuPS1, accumulation of HuPS1 derivatives is accompanied by a compensatory, and highly selective, decrease in the steady-state levels of murine PS1 and PS2 derivatives. Similarly, the levels of murine PS1 derivatives are diminished in cultured cells overexpressing HuPS2 (Thinakaran *et al.*, 1997a). Interestingly, overexpression of PS1 Δ E9 variant, which fails to be cleaved, also resulted in compromised accumulation of murine PS1/PS2 derivatives; thus the "replacement" of murine PS1/PS2 by overexpressed HuPS1 occurs independent of endoproteolysis (Lee *et al.*, 1997; Thinakaran *et al.*, 1997). These results are consistent with a model in

which the abundance of PS1 and PS2 derivatives are coordinately regulated by competition for limiting cellular factor(s) (Figure 1) (Thinakaran *et al.*, 1997).

CONSEQUENCES OF PS MUTATIONS ON ENDOPROTEOLYSIS

The metabolism of several PS1 variants containing FAD-linked missense mutations has been examined in cultured cells and in transgenic mice. In cultured cells, the PS1E9 variant failed to be cleaved (Thinakaran *et al.*, 1996), but the human M146L, H163R, A246E, E280A, L286V, L392V, or C410Y PS1 variants and the PS2 N141I variant are efficiently processed into NTF and CTF (Borchelt *et al.*, 1996; Citron *et al.*, 1997; Kim *et al.*, 1997a; Podlisny *et al.*, 1997; Tomita *et al.*, 1997). The HuPS1 M146L, M146V, H163R, A246E, and L286V variants are also efficiently cleaved into two fragments in brains of transgenic mice (Borchelt *et al.*, 1996; Duff *et al.*, 1996; Citron *et al.*, 1997; Lee *et al.*, 1997). Furthermore, PS1 variants I143T, G384A, and C410Y have been found to be cleaved in brains of individuals harboring these mutations (Hendriks *et al.*, 1997; Podlisny *et al.*, 1997). Hence, full-length FAD-linked variants with missense substitutions undergo endoproteolysis in a manner not unlike that described for wt PS1. Nevertheless, the accumulation of full-length PS1 has been reported in the brain in two individuals with FAD harboring a G209V PS1 mutation (Levey *et al.*, 1997).

Curiously, whereas HuPS1 derivatives in the brains of mice that express the A246E or M146L mutant PS1 accumulate to saturable levels and to 1:1 stoichiometry, there is a quantifiable ~1.5-fold increase in the levels of mutant derived fragments relative to those generated from wt HuPS1 (Lee *et al.*, 1997). Although parallel increases in levels of C-terminal fragments derived from mutant PS 1 may seem surprising (because both the A246E and M146L mutations reside in the N-terminal ~28-kD derivative), these observations are consistent with the hypothesis that the accumulation of PS1 NTF and CTF are coordinately regulated. It is not known whether the observed elevation in the “set-point” in accumulated levels of mutant PS-derived fragments is the result of enhanced endoproteolytic processing of mutant PS and/or greater stability of mutant PS 1-derived fragments is not known (Lee *et al.*, 1997). It is important to note that elevations in PS1 fragments or full-length PS1 have not been detected in brains of individuals harboring FAD-linked PS1 mutations (Hendriks *et al.*, 1997; Podlisny *et al.*, 1997). However, it is conceivable that confounding antemortem events or postmortem variables may mask relatively subtle differences in accumulated PS1 or its fragments in the brains of affected individuals. Nevertheless, recent studies have documented that, in brain extracts from an individual harboring the FAD-linked PS2 N141I mutation, the C-terminal derivative of PS2 accumulates to ~2-fold higher levels as compared to the C-terminal derivative in brain homogenates from three unaffected family members (Kim and Tanzi, personal communication).

Recently, overexpressed full-length PS1 and PS2, and endogenous CTFs have been shown to be cleaved by caspase-type proteases following induction of apoptosis by chemical inducers such as staurosporine or etoposide (Kim *et al.*, 1997b; Loetscher *et al.*, 1997). Cleavage of PS1/PS2 by a member of the apoptotic protease cascade raises the possibility that apoptosis-mediated cleavage of PS might have an important role in the pathogenesis of AD. Indeed, cultured neuroblastoma cells overexpressing FAD-linked N141IPS2 variant have been shown to produce higher levels of caspase-generated CTF (Kim *et al.*, 1997b). At present, it is not clear whether caspase cleavage of PS1/PS2 in *in vitro* paradigms of apoptosis reflects merely the sensitivity of these substrates to activated caspases

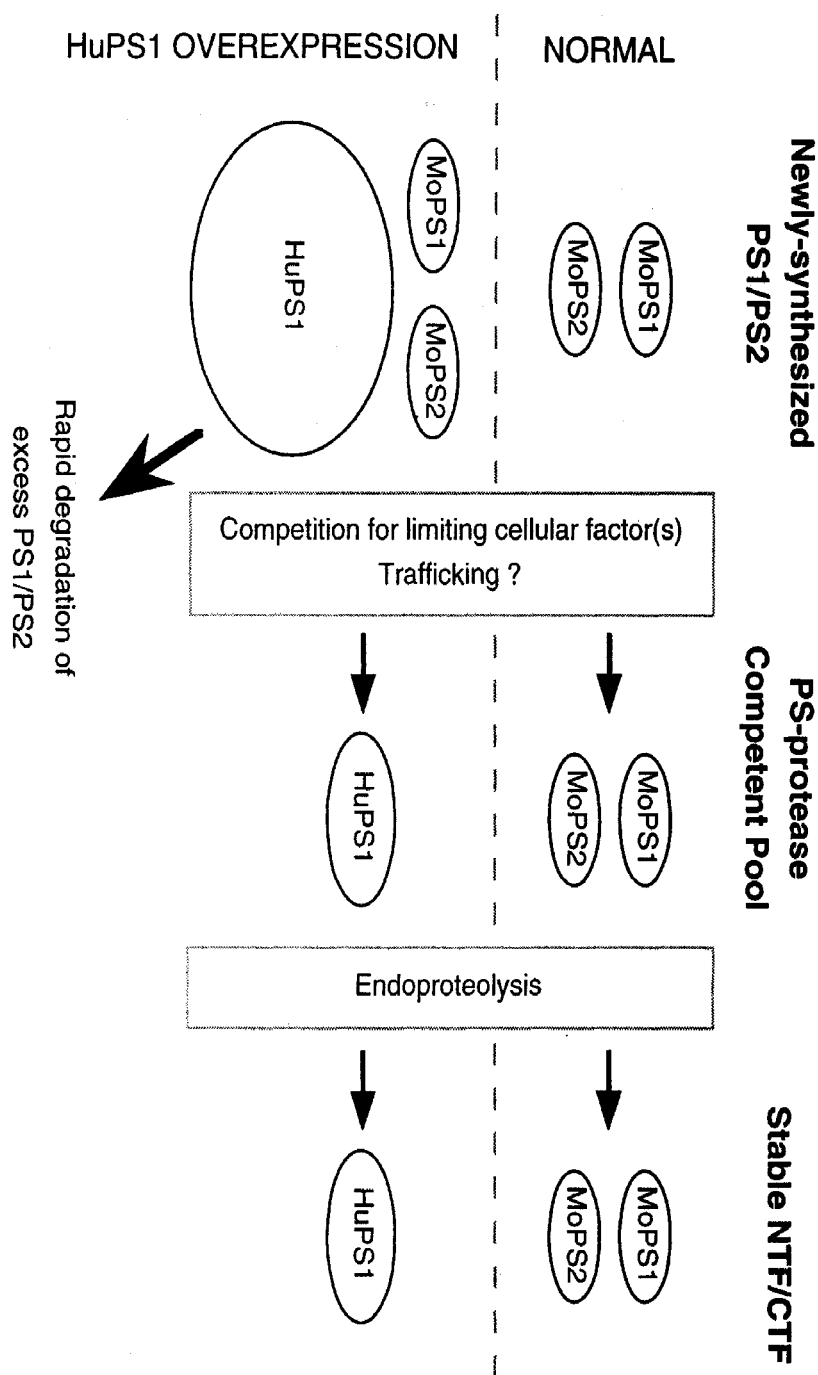


Figure 1. Model for PS1/PS2 processing. Nascent PS1/PS2 polypeptides are targeted to the cleavage pathway by successful interaction with limiting cellular factor(s). In transfected cells and transgenic mice, overexpressed HuPS1 competes with endogenous mouse (Mo) PS1/PS2 for these interactions. The "excess" PS1/PS2 polypeptides that are not targeted for cleavage pathway are rapidly degraded, whereas the processed derivatives are turned over with half lives of ~24 h (Ratovitski *et al.*, 1997; Podlisny *et al.*, 1997; Kim *et al.*, 1997).

or play an important role in the cell death pathway. For example, caspase-generated CTFs were not readily detectable in brains of transgenic mice expressing PS1 M146L, H163R, A246E, L286V, or Δ E9 variants (Borchelt *et al.*, 1996; Duff *et al.*, 1996; Citron *et al.*, 1997; Lee *et al.*, 1997). Furthermore, the levels of caspase-generated ~14 kDa PS1 CTF in brains of individuals harboring FAD linked PS1 C410Y mutation, or sporadic AD cases were indistinguishable from that of controls (Podlisny *et al.*, 1997). Thus, the relationship between caspase cleavage of PS1/PS2 to the pathophysiology of AD remains to be established.

BIOLOGICAL FUNCTIONS OF PS1

The first major insight regarding PS function emerged with the discovery of a homologous gene in *C. elegans*, termed *sel-12*; mutant alleles of *sel-12* were uncovered as suppressors of a multivulval phenotype in *C. elegans* mediated by gain of function alleles of the *C. elegans* Notch homologues *lin-12* and *glp-1* (Levitan and Greenwald, 1995). Notch and LIN-12/GLP-1 are transmembrane receptors required for the specification of cell fate and lateral inhibition during development (Artavanis-Tsakonas *et al.*, 1995). Although details regarding the molecular mechanisms by which SEL-12 facilitates signalling mediated by LIN-12 have not been established, two models have been proposed: first, SEL-12 could regulate LIN-12 trafficking and cell surface expression; or, second, SEL-12 could act in a signalling capacity to modulate pathways activated following the binding of cognate ligands to LIN-12. The extremely high amino acid homology between the PS and SEL-12, particularly in the first six transmembrane domains and the C-terminal ~90 residues, led to the prediction that related proteins would be functionally interchangeable. Consistent with this hypothesis, an egg-laying defect associated with loss of SEL-12 function in *C. elegans* is rescued efficiently by the expression of HuPS1 and -PS2; the rescue efficiency of HuPS was essentially indistinguishable to transgenic worms that express SEL-12 (Levitan *et al.*, 1996; Baumeister *et al.*, 1997). Notably, the egg-laying defect was only weakly rescued in transgenic worms that express several human FAD-linked PSI variants (Levitan *et al.*, 1996; Baumeister *et al.*, 1997), suggesting that PS1 missense variants behaved as loss-of-function alleles. Interestingly, the PS1 Δ E9 variant showed considerable rescue activity relative to other PS1 missense variants. Although the significance of this finding is unclear, it suggests that endoproteolytic cleavage is not obligatory for PS1 function in this developmental paradigm (Levitan *et al.*, 1996; Baumeister *et al.*, 1997).

Although the *C. elegans* rescue experiments provided compelling evidence that HuPS1 and -PS2 could substitute for SEL-12, the role of PS in mammalian development was uncertain. *In situ* hybridization studies and RT-PCR approaches in mouse embryos revealed that *PS1* is expressed in an ubiquitous manner during embryonic development (i.e., as early as embryonic day E8.5) (Lee *et al.*, 1996). However, the general spatial and temporal expression patterns of *PS* mRNA do not directly coincide with the expression patterns of any specific member of the known mammalian Notch homologs. These results suggested that, during mammalian development, PS1 function is not limited to Notch signalling alone (Lee *et al.*, 1996).

PS1 KNOCKOUT MICE

To examine the *in vivo* role of PS1 in mammalian development, mice with a targeted disruption of the PS1 gene were generated (Shen *et al.*, 1997; Wong *et al.*, 1997). Homozygous mutant mice failed to survive beyond the first ten minutes after birth. The most striking phenotype observed in *PS1*^{-/-} embryos was a severe perturbation in the development of the axial skeleton and ribs. The failed development of the axial skeleton in *PS1*^{-/-} animals was traced to defects in somitogenesis; in E8.5 and E9.5 embryos, somites were irregularly shaped and misaligned along the entire length of the neural tube and largely absent at the caudal most regions (Wong *et al.*, 1997). The abnormal somite patterns in *PS1*^{-/-} embryos are highly reminiscent of somite segmentation defects described in mice with functionally inactivated *Notch1* and *Dll1* (encoding a Notch ligand) alleles (Conlon *et al.*, 1995; Hrabe de Angelis *et al.*, 1997). Remarkably, the expression of mRNA that encodes Notch1 and Dll1 is reduced considerably in the presomitic mesoderm of *PS1*^{-/-} mice (Wong *et al.*, 1997). In addition, all *PS1*^{-/-} embryos exhibited intraparenchymal hemorrhages after day 11 of gestation. It has also been reported that, in the brains of *PS1*^{-/-} mice, the ventricular zone is thinner by day 14.5 and the massive neuronal loss in specific subregions is apparent after day 16.5. These observations have been interpreted to indicate that PS1 is required for normal neurogenesis and neuronal survival (Shen *et al.*, 1997). In the midst of the confounding cerebral hemorrhage, however, the neuronal phenotypes remain unsettled. A more satisfying model, in which the *PS1* gene is ablated in a conditional manner, is required to clarify this issue.

In view of the evidence in *C. elegans* which documented that FAD-linked mutant PS failed to completely rescue an egg-laying defect in worms lacking *sel-12*, very recently Wong and colleagues examined the ability of PS1 A246E variant to complement the embryonic lethality and axial skeletal defects in mice lacking PS1. The phenotypes observed in *PS1*^{-/-} mice were efficiently rescued by both wild type and A246E PS1 variant expressed using a mouse prion promoter (Thinakaran *et al.*, 1996), consistent with the view that FAD-linked PS1 mutants retain normal function during mammalian embryonic development (Davis *et al.*, 1998).

THE ROLE OF PS IN FAD AND A β METABOLISM

The mechanisms by which FAD-linked PS1 and PS2 variants cause FAD are unclear. Nevertheless, the incomplete rescue of egg-laying defect by FAD-linked PS1 variants suggests that these mutant PS act as loss-of-function alleles. However, the absence of nonsense or frameshift mutations that lead to truncated PS1/PS2 supports the notion that AD is caused not by the loss, but by the gain, of deleterious properties of mutant polypeptides. In this regard, studies have suggested that PS2 may participate in neuronal apoptosis (Wolozin *et al.*, 1996); the transient overexpression of full-length PS2 in nerve growth factor-differentiated PC12 cells led to a pertussis toxin-sensitive increase in apoptosis induced by trophic factor withdrawal or treatment with A β . Moreover, the expression of cDNA that encode antisense *PS2* mRNA protected against apoptotic cell death induced by trophic factor withdrawal or glutamate-induced cell death. In support of these investigations, PC12 cells that express the L286V mutant PS1 exhibit significant increases in oxidative stress, increased [Ca²⁺]_i following exposure to A β , and increased susceptibility to apoptosis induced by trophic factor withdrawal and A β (Guo *et al.*,

1997). Collectively, these studies suggest that the expression of mutant PS may influence neurodegeneration. On the other hand, studies of transgenic mice expressing mutant PS1 (Borchelt *et al.*, 1996; Duff *et al.*, 1996; Citron *et al.*, 1997; Lee *et al.*, 1997) have failed to disclose any cellular abnormalities consistent with apoptosis (see below).

The most provocative insights pertaining to the mechanisms by which mutant PS1 predispose carriers to FAD emerged initially from studies that examined the conditioned medium from fibroblasts or the plasma from affected members of pedigrees with PS1/PS2-linked mutations. Using highly sensitive sandwich ELIS A assays with antibodies specific for the C-terminus of A β 40 and A β 42 (Suzuki *et al.*, 1994), it became apparent that the ratio of A β 42(43)/A β 40 in affected individuals was significantly elevated relative to unaffected family members (Scheuner *et al.*, 1996). These data suggest that FAD-linked PS1/PS2 variants influence processing at the “ γ -secretase” site and cause AD by increasing the extracellular concentration of highly amyloidogenic A β 42(43) species, thus fostering A β deposition in the brain.

The influence of wt and mutant PS1 and PS2 on A β 40 and A β 42(43) production has been evaluated in transfected mammalian cells and the brains of transgenic mice. Sandwich ELISA analysis of the conditioned medium of cultured mammalian cells that coexpress human APP with either wt or mutant PS (Borchelt *et al.*, 1996; Citron *et al.*, 1997; Tomita *et al.*, 1997) have clearly documented that a variety of mutant PS1 variants, including A246E, M146L, β E9, L286V, L392V, and H163R, and the PS2 variant N141I, influence APP processing in a manner that elevates levels of A β 42(43) relative to cells expressing wt PS. In a variety of cultured mammalian cells expressing mutant PS1/PS2, the ratio of A β 42(43)/A β 40 was elevated by ~1.5–3 fold.

In the brains of young (2–3 month) transgenic mice that express HuPS1 harboring FAD-linked mutations, the ratio of A β 42/A β 40 was increased (Borchelt *et al.*, 1996; Duff *et al.*, 1996; Citron *et al.*, 1997). In recent studies, Borchelt and colleagues reported that transgenic mice coexpressing A246E HuPS 1 and a chimeric mouse/human APP695 (Borchelt *et al.*, 1996) harboring a human A β domain as well as mutations (K595N, M596L) linked to APPswe FAD pedigrees, contained numerous amyloid deposits at 12 months of age (Borchelt *et al.*, 1997). Many of these deposits were associated with dystrophic neurites and reactive astrocytes. Parallel analyses of brains from age-matched animals that express APPswe alone or mice that express A246E HuPS1 alone were free of amyloid deposits. These data convincingly demonstrate that the A246E HuPS1 acts synergistically with APPswe to accelerate the rate of amyloid deposition. Neither overt behavioral (up to 16 months) nor neuropathological (up to 12 months) alterations have been observed in mice that express A246E HuPS1 alone. These findings suggest that the principal mechanism by which mutations in PS1 cause disease is through elevating extracellular concentrations of A β 42 and, thereby, accelerating the deposition of amyloid.

The biochemical mechanism(s) by which mutant PS1 elevates A β 42 levels is not clearly understood. Two recent reports documented that full-length PS1 and PS2 form stable heteromeric assemblies with APP in cultured mammalian cells (Weidemann *et al.*, 1997; Xia *et al.*, 1997). In contrast, we failed to provide evidence for physiological interaction between PS1/PS2 and APP (Thinakaran *et al.*, 1998), under conditions where APP can be co-isolated with a known interacting protein, Fe65 (Fiore *et al.*, 1995; Guénette *et al.*, 1996). Furthermore, PS1 was not cross-linked to APP under conditions where PS1 derivatives were efficiently cross-linked to each other. These latter results raise the possibility that mutant PS1/PS2 effects on APP metabolism may not be mediated through

direct physical associations. Whether mutant PS1/PS2 influences protein trafficking in a manner that promotes co-compartmentalization of putative “ γ -42-secretase(s)” and their substrates (i.e., APP or its potentially amyloidogenic C-terminal derivatives) remains to be established.

Finally, to address whether the selective elevation in the levels of A β 42 in plasma of individuals with FAD-linked PS1 mutations may be due to a loss of normal PS function, Wong and colleagues assayed A β 40 and A β 42 levels in brains of mice lacking one *PS1* allele. The levels of A β 42 and A β 40 in *PS1*^{+/-} mice were indistinguishable from that of control mice (Davis *et al.*, 1998). Furthermore, neuronal cultures derived from *PS1*^{-/-} neurons fail to secrete A β (De Strooper *et al.*, 1998). These studies support the idea that mutant PS1 cause AD not by loss of normal function, but by acquiring property(ies) that elevate A β 42 production.

REFERENCES

- Alzheimer's Disease Collaborative Group (1995) The structure of the presenilin 1 (*S182*) gene and identification of six novel mutations in early onset AD families. *Nature Genetics*, **11**, 219–222.
- Artavanis-Tsakonas, S., Matsuno, K., and Fortini, M.E. (1995) Notch signaling. *Science*, **268**, 225–232.
- Baumeister, R., Leimer, U., Zweckbronner, I., Jakubek, C., Grünberg, J., and Haass, C. (1997) Human presenilin-1, but not familial Alzheimer's disease (FAD) mutants, facilitate *Caenorhabditis elegans* Notch signalling independently of proteolytic processing. *Genes & Function*, **1**, 149–159.
- Benkovic, S.A., McGowan, E.M., Rothwell, N.J., Hutton, M., Morgan, D.G., and Gordon, M.N. (1997) Regional and cellular localization of presenilin-2 RNA in rat and human brain. *Exp. Neurol.*, **145**, 555–564.
- Boissière, F., Pradier, L., Delaère, P., Faucheux, B., Revah, F., Brice, A., Agid, Y., and Hirsch, B.C. (1996) Regional and cellular presenilin 2 (STM2) gene expression in the human brain. *Neuroreport*, **7**, 2021–2025.
- 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 1–42/1–40 ratio in vitro and *in vivo*. *Neuron*, **17**, 1005–1013.
- Borchelt, D.R., Ratovitski, T., Van Lare, J., Lee, M.K., Gonzales, V.B., Jenkins, N.A., Copeland, N.G., Price, D.L., and Sisodia, S.S. (1997). Accelerated amyloid deposition in the brains of transgenic mice co-expressing mutant presenilin 1 and amyloid precursor proteins. *Neuron*, **19**, 939–945.
- Busciglio, J., Hartmann, H., Lorenzo, A., Wong, C., Baumann, K., Sommer, B., Staufenbiel, M., and Yankner, B.A. (1997) Neuronal localization of presenilin-1 and association with amyloid plaques and neurofibrillary tangles in Alzheimer's disease. *J. Neurosci.*, **17**, 5101–5107.
- Citron, M., Westaway, D., Xia, W., Carlson, G., Diehl, T., Levesque, G., Johnson-Wood, K., Lee, M., Seubert, P., Davis, A., Kholodenko, D., Motter, R., Sherrington, R., Perry, B., Yao, H., Strome, R., Lieberburg, I., Rommens, J., Kim, S., Schenk, D., Fraser, P., St.George Hyslop, P., and Selkoe, D.J. (1997) Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid β -protein in both transfected cells and transgenic mice. *Nature Med.*, **3**, 67–72.
- Conlon, R.A., Reaume, A.G., and Rossant, J. (1995) *Notch 1* is required for the coordinate segmentation of somites. *Development*, **121**, 1533–1545.

- Cook, D.G., Sung, J.C., Golde, T.E., Felsenstein, K.M., Wojczyk, B.S., Tanzi, R.E., Trojanowski, J.Q., Lee, V.M.-Y., and Doms, R.W. (1996) Expression and analysis of presenilin 1 in a human neuronal system: localization in cell bodies and dendrites. *Proc. Natl. Acad. Sci. USA*, **93**, 9223–9228.
- Cribbs, D.H., Chen, L.S., Bende, S.M., and LaFerla, F.M. (1996) Widespread neuronal expression of the presenilin-1 early-onset Alzheimer's disease gene in the murine brain. *Am. J. Pathol.*, **148**, 1797–1806.
- Davis, J.A., Naruse, S., Chen, H., Eckman, C., Younkin, S., Price, D.L., Borchelt, D.R., Sisodia, S.S., and Wong, P.C. (1988) An Alzheimer's disease-linked PS1 variant rescues the developmental abnormalities of *PS1*-deficient embryos. *Neuron*, **20**, 603–609.
- De Strooper, B., Beullens, M., Contreras, B., Levesque, L., Craessaerts, K., Cordell, B., Moechars, D., Bollen, M., Fraser, P., St. George Hyslop, P., and Vanleuven, F. (1997) Phosphorylation, subcellular localization, and membrane orientation of the Alzheimer's disease-associated presenilins. *J. Biol. Chem.*, **272**, 3590–3598.
- De Strooper, B., Saltig, P., Craessaerts, R., Vanderstichele, H., Guhde, G., Annaert, W., Figura, K.V., and van Leuven, F. (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature*, **391**, 387–390.
- Dewji, N.N., and Singer, S.J. (1997) Cell surface expression of the Alzheimer disease-related presenilin proteins. *Proc. Natl. Acad. Sci. USA*, **94**, 9926–9931.
- Doan, A., Thinakaran, G., Borchelt, D.R., Slunt, H.H., Ratovitsky, T., Podlisny, M., Selkoe, D.J., Seeger, M., Gandy, S.E., Price, D.L., and Sisodia, S.S. (1996) Protein topology of presenilin 1. *Neuron*, **17**, 1023–1030.
- Duff, K., Eckman, C., Zehr, C., Yu, X., Prada, C.-M., Perez-Tur, J., Hutton, M., Buee, L., Harigaya, Y., Yager, D., Morgan, D., Gordon, M.N., Holcomb, L., Refolo, L., Zenk, B., Hardy, J., and Younkin, S. (1996) Increased amyloid- β 42(43) in brains of mice expressing mutant presenilin 1. *Nature*, **383**, 710–713.
- Fiore, F., Zambrano, N., Minopoli, G., Donini, V., Duilio, A., and Russo, T. (1995) The regions of the FE65 protein homologous to the phosphotyrosine interaction/phosphotyrosine binding domain of Shc bind the intracellular domain of the Alzheimer's amyloid precursor protein. *J. Biol. Chem.*, **270**, 30853–30856.
- Gu  nette, S., Chen, J., Jondro, P.D., and Tanzi, R.E. (1996) Association of a novel human FE65-like protein with the cytoplasmic domain of the β -amyloid precursor protein. *Proc. Natl. Acad. Sci. USA*, **93**, 10832–10837.
- Guo, Q., Sopher, B.L., Furukawa, K., Pham, D.G., Robinson, N., Martin, G.M., and Mattson, M.P. (1997) Alzheimer's presenilin mutation sensitizes neural cells to apoptosis induced by trophic factor withdrawal and amyloid β -peptide: involvement of calcium and oxyradicals. *J. Neurosci.*, **17**, 4212–4222.
- Hendriks, L., Thinakaran, G., Harris, C.L., De Jonghe, C., Martin, J.-J., Sisodia, S.S., and Van Broeckhoven, C. (1997). Processing of presenilin 1 in brains of Alzheimer's disease patients and controls. *Neuroreport*, **8**, 1717–1721.
- Hrabe de Angelis, M., McIntyre, J., and Gossler, A. (1997) Maintenance of somite borders in mice requires the *Delta* homologue *Dll1*. *Nature*, **386**, 717–721.
- Kim, T.-W., Pettingell, W.H., Hallmark, O.G., Moir, R.D., Wasco, W., and Tanzi, R.E. (1997a) Endoproteolytic cleavage and proteasomal degradation of presenilin 2 in transfected cells. *J. Biol. Chem.*, **272**, 11006–11010.
- Kim, T.-W., Pettingell, W.H., Jung, Y.-K., Kovacs, D.M., and Tanzi, R.E. (1997b) Alternative cleavage of Alzheimer-associated presenilins during apoptosis by a caspase-3 family protease. *Science*, **277**, 373–376.
- Kovacs, D.M., Fausett, H.J., Page, K.J., Kim, T.-W., Moir, R.D., Merriam, D.E., Hollister, R.D., Hallmark, O.G., Mancini, R., Felsenstein, K.M., Hyman, B.T., Tanzi, R.E., and Wasco, W. (1996) Alzheimer-associated presenilins 1 and 2: neuronal expression in brain and localization to intracellular membranes in mammalian cells. *Nature Med.*, **2**, 224–229.

- Lah, J.J., Heilman, C.J., Nash, N.R., Rees, H.D., Yi, H., Counts, S.E., and Levey, A.I. (1997) Light and electron microscopic localization of presenilin-1 in primate brain. *J Neurosci.*, **17**, 1971–1980.
- Lee, M.K., Slunt, H.H., Martin, L.J., Thinakaran, G., Kim, G., Gandy, S.E., Seeger, M., Koo, E., Price, D.L., and Sisodia, S.S. (1996) Expression of presenilin 1 and 2 (PS1 and PS2) in human and murine tissues. *J. Neurosci.*, **16**, 7513–7525.
- Lee, M.K., Borchelt, D.R., Kim, G., Thinakaran, G., Slunt, H.H., Ratovitski, T., Martin, L.J., Kittur, A., Gandy, S., Levey, A.I., Jenkins, N., Copeland, N., Price, D.L., and Sisodia, S.S. (1997) Hyperaccumulation of FAD-linked presenilin 1 variants *in vivo*. *Nature Med.*, **3**, 756–760.
- Lehmann, S., Chiesa, R., and Harris, D.A. (1997) Evidence for a six-transmembrane domain structure of presenilin 1. *J. Biol. Chem.*, **272**, 12047–12051.
- Levey, A.I., Heilman, C.J., Lah, J.J., Nash, N.R., Rees, H.D., Wakai, M., Mirra, S.S., Rye, D.B., Nochlin, D., Bird, T.D., and Mufson, E.J. (1997). Presenilin-1 protein expression in familial and sporadic Alzheimer's disease. *Ann. Neurol.*, **41**, 742–753.
- Levitan, D., Doyle, T.G., Brousseau, D., Lee, M.K., Thinakaran, G., Slunt, H.H., Sisodia, S.S., and Greenwald, I. (1996) Assessment of normal and mutant human presenilin function in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA*, **93**, 14940–14944.
- Levitan, D., and Greenwald, I. (1995) Facilitation of *lin-12*-mediated signalling by *sel-12*, a *Caenorhabditis elegans* S182 Alzheimer's disease gene. *Nature*, **377**, 351–354.
- Li, J., Xu, M., Zhou, H., Ma, J., and Potter, H. (1997) Alzheimer presenilins in the nuclear membrane, interphase kinetochores, and centrosomes suggest a role in chromosome segregation. *Cell*, **90**, 917–927.
- Li, X., and Greenwald, I. (1996) Membrane topology of the *C. elegans* SEL-12 presenilin. *Neuron*, **17**, 1015–1021.
- Loetscher, H., Deuschle, U., Brockhaus, M., Reinhardt, D., Nelboeck, P., Mous, J., Grünberg, J., Haass, C., and Jacobsen, H. (1997) Presenilins are processed by caspase-type proteases. *J. Biol. Chem.*, **272**, 20655–20659.
- Mercken, M., Takahashi, H., Honda, T., Sato, K., Murayama, M., Nakazato, Y., Noguchi, K., Imahori, K., and Takashima, A. (1996) Characterization of human presenilin 1 using N-terminal specific monoclonal antibodies: Evidence that Alzheimer mutations affect proteolytic processing. *FEBS Lett.*, **389**, 297–303.
- Moussaoui, S., Czech, C., Pradier, L., Blanchard, V., Bonici, B., Gohin, M., Imperato, A., and Revah, F. (1996) Immunohistochemical analysis of presenilin-1 expression in the mouse brain. *FEBS Lett.*, **383**, 219–222.
- Page, K., Hollister, R., Tanzi, R.E., and Hyman, B.T. (1996) *In situ* hybridization analysis of presenilin 1 mRNA in Alzheimer disease and in lesioned rat brain. *Proc. Natl. Acad. Sci. USA*, **93**, 14020–14024.
- Podlisny, M.B., Citron, M., Amarante, P., Sherrington, R., Xia, W., Zhang, J., Diehl, T., Levesque, G., Fraser, P., Haass, C., Koo, E.H.M., Seubert, P., StGeorge-Hyslop, P., Teplow, D.B., and Selkoe, D.J. (1997) Presenilin proteins undergo heterogeneous endoproteolysis between Thr₂₉₁ and Ala₂₉₉ and occur as stable N- and C-terminal fragments in normal and Alzheimer brain tissue. *Neurobiol. Dis.*, **3**, 325–337.
- Ratovitski, T., Slunt, H.H., Thinakaran, G., Price, D.L., Sisodia, S.S., and Borchelt, D.R. (1997) Endoproteolytic processing and stabilization of wild-type and mutant presenilin. *J. Biol. Chem.*, **272**, 24536–24541.
- Rogaev, E.I., Sherrington, R., Rogaeva, E.A., Levesque, G., Ikeda, M., Liang, Y., Chi, H., Lin, C., Holman, K., Tsuda, T., Mar, L., Sorbi, S., Nacmias, B., Piacentini, S., Amaducci, L., Chumakov, I., Cohen, D., Lannfelt, L., Fraser, P.E., Rommens, J.M., and St George-Hyslop, P.H. (1995) Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. *Nature*, **376**, 775–778.
- Sahara, N., Yahagi, Y., Takagi, H., Kondo, T., Okochi, M., Usami, M., Shirasawa, T., and Mori, H. (1996) Identification and characterization of presenilin I-467, I-463 and I-374. *FEES Lett.*, **381**, 7–11.

- Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T.D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-Lahad, E., Viitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wasco, W., Lannfelt, L., Selkoe, D., and Younkin, S. (1996) Secreted amyloid β -protein similar to that in the senile plaques of Alzheimer's disease is increased *in vivo* by the presenilin 1 and 2 and *APP* mutations linked to familial Alzheimer's disease. *Nature Med.*, **2**, 864–852.
- Seeger, M., Nordstedt, C., Petanceska, S., Kovacs, D.M., Gouras, G.K., Hahne, S., Fraser, P., Levesque, L., Czernik, A.J., St. George-Hyslop, P., Sisodia, S.S., Thinakaran, G., Tanzi, R.E., Greengard, P., and Gandy, S. (1997) Evidence for phosphorylation and oligomeric assembly of presenilin 1. *Proc. Natl. Acad. Sci. USA*, **94**, 5090–5094.
- Shen, J., Bronson, R.T., Chen, D.F., Xia, W., Selkoe, D.J., and Tonegawa, S. (1997) Skeletal and CNS defects in *presenilin-1*-deficient mice. *Cell*, **89**, 629–639.
- Sherrington, R., Rogaev, E.I., Liang, Y., Rogaeva, E.A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., Tsuda, T., Mar, L., Foncin, J.-E., Bruni, A.C., Montesi, M.P., Sorbi, S., Rainero, I., Pinessi, L., Nee, L., Chumakov, I., Pollen, D., Brookes, A., Sanseau, P., Polinsky, R.J., Wasco, W., Da Silva, H.A.R., Haines, J.L., Pericak-Vance, M.A., Tanzi, R.E., Roses, A.D., Fraser, P.E., Rommens, J.M., and St George-Hyslop, P.H. (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature*, **375**, 754–760.
- Slunt, H.H., Thinakaran, G., Lee, M.K., and Sisodia, S.S. (1995) Nucleotide sequence of the chromosome 14-encoded *S182* cDNA and revised secondary structure prediction. *Amyloid: Int. J. Exp. Clin. Invest.*, **2**, 188–190.
- Suzuki, N., Cheung, T.T., Cai, X.-D., Odaka, A., Otvos, L., Jr., Eckman, C., Golde, T.E., and Younkin, S.G. (1994) An increased percentage of long amyloid β protein secreted by familial amyloid β protein precursor (β APP₇₁₇) mutants. *Science*, **264**, 1336–1340.
- Suzuki, T., Nishiyama, K., Murayama, S., Yamamoto, A., Sato, S., Kanazawa, I., and Sakaki, Y. (1996) Regional and cellular *presenilin 1* gene expression in human and rat tissues. *Biochem. Biophys. Res. Commun.*, **219**, 708–713.
- Thinakaran, G., Borchelt, D.R., Lee, M.K., Slunt, H.H., Spitzer, L., Kim, G., Ratovitski, T., Davenport, E., Nordstedt, C., Seeger, M., Hardy, J., Levey, A.I., Gandy, S.E., Jenkins, N., Copeland, N., Price, D.L., and Sisodia, S.S. (1996) Endoproteolysis of presenilin 1 and accumulation of processed derivatives *in vivo*. *Neuron*, **17**, 181–190.
- Thinakaran, G., Harris, C.L., Ratovitski, T., Davenport, F., Slunt, H.H., Price, D.L., Borchelt, D.R., and Sisodia, S.S. (1997). Evidence that levels of presenilins (PS1 and PS2) are coordinately regulated by competition for limiting cellular factors. *J. Biol. Chem.*, **272**, 28415–28422.
- Thinakaran, G., Regard, J.B., Bouton, C.M.L., Harris, C.L., Sabo, S., Price, D.L., Borchelt, D.R., and Sisodia, S.S. Stable association of presenilin derivatives and absence of presenilin interactions with APP. (1998) *Neurobiol Dis.*, **4**, 438–453.
- Tomita, T., Maruyama, K., Saido, T.C., Kume, H., Shinozaki, K., Tokuhira, S., Capell, A., Walter, J., Gruenberg, J., Haass, C., Iwatsubo, T., and Obata, K. (1997) The presenilin 2 mutation (N141I) linked to familial Alzheimer disease (Volga German families) increases the secretion of amyloid β protein ending at the 42nd (or 43rd) residue. *Proc. Natl. Acad. Sci. USA*, **94**, 2025–2030.
- Walter, J., Grünberg, J., Capell, A., Pesold, B., Schindzielorz, A., Citron, M., Mendl, K., St George-Hyslop, P., Multhaup, G., Selkoe, D.J., and Haass, C. (1997) Proteolytic processing of the Alzheimer disease-associated presenilin-1 generates an *in vivo* substrate for protein kinase C. *Proc. Natl. Acad. Sci. USA*, **94**, 5349–5354.
- Weidemann, A., Paliga, K., Dürrwang, U., Czech, C., Evin, G., Masters, C.L., and Beyreuther, K. (1997) Formation of stable complexes between two Alzheimer's disease gene products: presenilin-2 and β -amyloid precursor protein. *Nature Med.*, **3**, 328–332.

- Wolozin, B., Iwasaki, K., Vito, P., Ganjei, J.K., Lacanà, E., Sunderland, T., Zhao, B.Y., Kusiak, J.W., Wasco, V., and D'Adamio, L. (1996) Participation of presenilin 2 in apoptosis: enhanced basal activity conferred by an Alzheimer mutation. *Science*, **274**, 1710–1713.
- Wong, P.C., Zheng, H., Chen, H., Becher, M.W., Sirinathsinghji, D.J.S., Trumbauer, M.E., Chen, H.Y., Price, D.L., Van der Ploeg, L.H.T., and Sisodia, S.S. (1997) Presenilin 1 is required for *Notch1* and *Dll1* expression in the paraxial mesoderm. *Nature*, **387**, 288–292.
- Xia, W., Zhang, J., Perez, R., Koo, E.H., and Selkoe, D.J. (1997) Interaction between amyloid precursor protein and presenilins in mammalian cells: implications for the pathogenesis of Alzheimer disease. *Proc. Natl Acad. Sci. USA*, **94**, 8208–8213.
- Zhou, J., Liyanage, U., Medina, M., Ho, C., Simmons, A.D., Lovett, M., and Koski, K.S. (1997) Presenilin 1 interaction in the brain with a novel member of the armadillo family. *Neuroreport*, **8**, 2085–2090.

11.

MOLECULAR BIOLOGY OF PRESENILIN 2

WILMA WASCO

*Genetics and Aging Unit, Department of Neurology, Massachusetts General Hospital,
Harvard Medical School, Boston, MA*

INTRODUCTION

The presenilins constitute an evolutionarily conserved family of ubiquitously expressed, multiple transmembrane domain proteins. To date, the genes for two mammalian presenilin proteins, termed presenilin 1 (PS1) and presenilin 2 (PS2) have been identified and localized to human chromosome 14 (Sherrington *et al.*, 1995) and chromosome 1 (Levy-Lehad *et al.*, 1995; Rogeav *et al.*, 1995; Li *et al.*, 1995) respectively. Interestingly, although the proteins encoded by the two genes are highly homologous and have an overall amino acid identity of 63% (Levy-Lehad *et al.*, 1995, Rogeav *et al.*, 1995), intensive mutation and epidemiological analyses has revealed some interesting differences between the two proteins. The number of identified mutations in PS1 is far greater than those in PS2. More than 50 individual point mutations in PS1 have been identified and found to be responsible for the majority of early-onset familial Alzheimer's disease. All but one of these are point mutations that result in single amino acid changes. In contrast, only two mutations in PS2, both of which are point mutations, have been identified and linked to kindred-specific forms of the disease. The first PS2 mutation to be identified results in an asparagine to isoleucine change at amino acid 141 (N141I) and is responsible for the disease in a large Volga-German kindred (Levy-Lehad *et al.*, 1995). The second PS2 mutation causes a methionine to valine change at amino acid 239 (M239V) and was identified in an Italian pedigree (Rogeav *et al.*, 1995). Whether the preponderance of PS1 verses PS2 mutations is due to the differing genomic environment of the two genes or to factors related to differing biological function of the two molecules remains unclear. Mutation analysis of the two proteins has also lead to the identification of a phenotypic difference. Generally, mutations in PS1 result in a relatively early and constant age-of-onset while mutations in PS2 lead to a somewhat later and more variable age-of-onset (Bird *et al.*, 1996). The recent identification of a PS1 asparagine to isoleucine disease-associated mutation at amino acid 135, a position and alteration that corresponds to the PS2 Volga German mutation, and the finding that the affected members of this pedigree have a relatively constant age-of-onset suggests that the observed variations in age-of-onset are dependent on the molecule itself as opposed to the specific position or nature of the amino acid alteration (Crook *et al.*, 1997).

Although the normal function(s) of presenilin 1 and 2 remain unknown, it is clear that the mammalian proteins are members of an evolutionarily conserved gene family. Presenilin homologues have been identified in *C. Elegans* (Levitan and Greenwald 1995; L'Hernault and Arduengo 1992), *Drosophila* (Hong and Koo 1997; Boulianne *et al.*, 1997) and *Xenopus*. (Tsujimura *et al.*, 1997). While little is known about the function of the homologues in *Drosophila* or *Xenopus*, the two *C. elegans* proteins, termed SEL-12 and SPE-4, are both believed to be involved in the trafficking and/or sorting of intracellular molecules. Mutation analyses of these proteins indicates that SEL-12 modulates signaling in the LIN-12/Notch mediated pathway and that SPE-4 is involved in intracellular protein sorting and trafficking during spermatogenesis. The ability of the mammalian presenilin proteins to rescue the SEL-12 phenotype in *C. elegans* (Levitan *et al.*, 1996) is an intriguing finding which indicates that clues to the biological role of the mammalian presenilins and to the mechanism(s) by which the FAD-associated mutations may ultimately come from a clearer understanding of the function of the presenilin homologues in organisms that are easily manipulated at the genetic level.

STRUCTURE OF PRESENILIN 2

Current data indicates that PS2 is encoded by a twelve exon gene that spans 23,737 base pairs (Levy-Lehad *et al.*, 1996; Prihar *et al.*, 1996,) and is located on the long arm of chromosome 1 at Iq42 (Levy-Lehad *et al.*, 1996; Takano *et al.*, 1997). The sequence of the PS2 cDNAs isolated from brain predict the translation results in the production a 448 amino acid polypeptide. The initiating methionine for the open reading frame of PS2 is located within exon 3, suggesting that the protein is encoded by only 10 of the 12 exons and that the first two exons contain noncoding information. The overall organization of the PS2 gene is strikingly similar to that of the PS1 gene (Hutton *et al.*, 1996). The majority of the intron-exon boundaries and the open reading frame are conserved between the two genes, although it is not clear whether a recently identified 5' non coding exon in PS1 is also present in PS2 (Rogeev *et al.*, 1997). At the present time, little information concerning the regulatory elements present in the PS2 promoter and/or the similarity of this region to the PS1 promoter (Mitsuda *et al.*, 1997; Rogeev *et al.*, 1997) is available.

There is considerable debate concerning the membrane topology of the presenilins. Although topological analyses have centered on PS1, the extreme similarity of PS2 to PS1 makes it probable that the two proteins take up similar positions within the membrane and for the purposes of this discussion it will be assumed that the two proteins have similar topologies. The original analysis of hydropathy plots, which indicate that both PS1 and PS2 have ten hydrophobic domains, predicted that six to nine of these domains actually spanned the membrane. Originally, the favored model was a seven transmembrane domain which predicted that the N- and C-termini were on opposite on the side of the membrane and that there was a large hydrophilic loop located between TMD 6 and 7. Subsequently, the analyses from three groups, two of which focus on PS1 (Doan *et al.*, 1996; Lehmann *et al.*, 1997) and one on SEL 12 (Li and Greenwald 1996), a *C.elegans* presenilin homologue, have refined the original predictions. All three of these groups assume that the presenilins are located in the intracellular membranes of the ER and Golgi and that consequently, the hydrophilic regions of the molecules are either cytoplasmic or lumenal. These studies utilized constructs that produce a series of presenilin or SEL-12 fusion proteins in combination with enzymatic assays and immunochemical

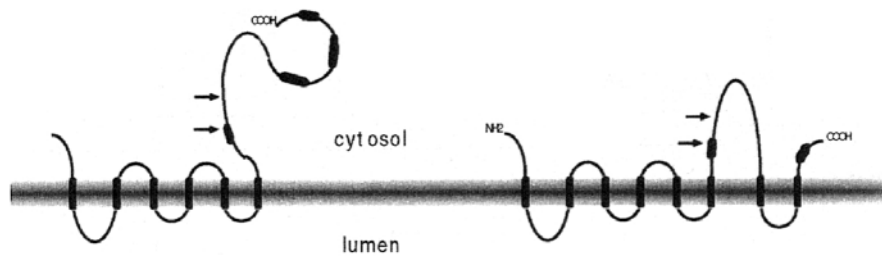


Figure 1. Six- and Eight-Transmembrane Domain Presenilin Topology Models. Two models for presenilin topology are presented. Arrows indicate the approximate position of presenilin 2 cleavage sites as discussed in the text.

analysis to determine the number of the hydrophobic domains that actually span the membrane and which side of the membrane (cytoplasmic or luminal) specific domains are situated. The results of all three studies indicate that the first six hydrophobia regions span the membrane, a finding that is in agreement with the original model. The SEL-12 analysis goes on to predict that while the 8th and 9th hydrophobic domains also span the membrane, the 7th and 10th do not (Li and Greenwald 1997). These findings suggest an eight-TMD model. The results of a similar study of presenilin 1 concluded that there were either six- or eight-TMD, but did not allow for differentiation between the two possibilities (Doan *et al.*, 1996). The most recent PS1 topology study predicts that only the first six hydrophobic domains span the membrane and that the last four hydrophobic domains remain on the cytoplasmic side of the membrane to create a relative large C-terminal domain (Lehmann *et al.*, 1997). An important consequence of this model is the loss of the large hydrophilic loop that is a prominent characteristic of the other models. This domain becomes part of a larger C-terminal domain in the six-TMD model (See Figure 1). Notably, each of the studies conclude that the N- and C-termini are on the cytoplasmic side of the membrane, that a 32 amino acid loop located between TMD 1 and 2 is on the luminal side of the membrane, and the remainder of the hydrophilic loops located before TMD6 are relatively small, ranging from only 5 to 9 amino acids. Accordingly, proteins that interact with PS2 in the cytoplasm have been predicted to do so by interaction with the N- or C-termini or with the large hydrophilic loop, while interactions with proteins located within the lumen of the ER/Golgi would most likely do so via the smaller TMD 1/2 hydrophilic loop.

PRESENILIN 2 EXPRESSION AND LOCALIZATION

Although PS2 is expressed to varying degrees in all tissues examined, the levels of expression are particularly low in brain and relatively high in skeletal muscle, heart and pancreas (Levy-Lehad *et al.*, 1995, 1996; Lee *et al.*, 1996). Despite the similarity of the PS1 and PS2 genes and proteins, Northern blot and in situ hybridization studies indicate that the levels of PS2 expression are much lower than those of PS1. Northern blot analysis indicates that the PS2 gene produces two major transcripts of 2.3 and 2.6 kb however, only the cDNA corresponding to the smaller message has been isolated. Therefore the exact relationship of the two PS2 transcripts remains unclear. Interestingly, while the smaller transcript is detected in all tissues examined, the larger transcript is detected only in heart, skeletal muscle and pancreas where it is far more abundant than the smaller message. While the identity of the 2.6 kb

message remains unknown, its differential expression raises the possibility of tissue specific expression of particular transcripts of PS2.

RT-PCR studies utilizing RNA derived from a variety of normal tissues (heart, brain, liver, lung, placenta and skeletal muscle) indicate the presence of rare alternatively spliced PS2 products which include species with in-frame deletions of exon 8 and simultaneous omissions of exons 3 and 4 (Prihar *et al.*, 1996). The splicing events of exons 3/4 and exon 8 appear to be independent events which occurred to a minor extent in all of the tissues examined. Exon 8 is predicted to encode 32 amino acids that comprise the latter part of transmembrane domain 6 and the first part of the large hydrophobic loop. The alternate splicing of exons 3 and 4 would appear to be an unusual event given that these mRNA's would lack the initiator methionine, and would suggest that if these transcripts are translated that they would begin at an internal methionine. Interestingly, the methionine located at codon 145 is in good agreement with the consensus sequence for translation initiation and it has been suggested that if these transcripts are functional, that this methionine might be utilized (Prihar *et al.*, 1996). A detailed expression analysis of these putative transcripts and their translation products awaits production of the appropriate immunologic reagents.

In situ hybridization and immunohistochemical studies indicate that within the brain message for PS2 is primarily detectable in neurons, and particularly in somal cytoplasm (Boissiere *et al.*, 1996; Deng *et al.*, 1996; Kovacs *et al.*, 1996; Lee *et al.*, 1996; McMillian *et al.*, 1996; Berezovska *et al.*, 1997; Benovic *et al.*, 1997; Blanchard *et al.*, 1997). These studies found that relatively intense staining signal was most commonly found in large pyramidal neurons, and that moderate or faint staining was usually present in smaller neurons. It is also clear that the expression of PS2 is not limited to the neuronal populations that are known to degenerate in Alzheimer's disease.

Although there have been reports of PS2 localization at the cell surface (Dwijen and Singer, 1997) and at the nuclear membrane (Li *et al.*, 1997) there is general agreement that the primary subcellular localization of both endogenous and transfected PS2 is within the intracellular membranes of the endoplasmic reticulum (ER) and the Golgi complex (Kovacs *et al.*, 1996; Walter *et al.*, 1996; Cook *et al.*, 1996). In neuronal cell lines PS1 staining has also been observed in dendrites, but not in axons (Cook *et al.*, 1996). These findings are supported by the results of a recent immunoelectron microscopy study of primate brain where PS1 was found to be associated with the cytoplasmic face of the ER/ Golgi intermediate compartment as well as in certain coated transport vesicles and dendrites (Lah *et al.*, 1997).

PRESENILIN 2 PROTEIN PROCESSING

The levels of PS2 expression are generally low enough to make detection of the protein in untransfected cells relatively difficult (Tomita *et al.*, 1997), however findings from a number of laboratories indicate that PS2 has a tendency to form high molecular weight aggregates, and that endogenous and transfected human PS2 is usually expressed as a closely migrating doublet polypeptide of 50–55 kD. In addition, it appears PS2 is similar to PS1 in that cleavage within the large hydrophilic loop generates two proteolytic fragments (Thinakerin *et al.*, 1996; Kim *et al.*, 1997a,b).

Studies utilizing cultured cells which have been transiently or stably transfected with wild-type or N141I mutant PS2 indicate that the FAD-associated mutation in the molecule has no obvious effect on

the production or processing of the protein (Kovacs *et al.*, 1996; Walter *et al.*, 1996; Tomika *et al.*, 1997.). Interestingly, an analysis of the effects of this same mutation in a tetracycline-regulated inducible cell system did reveal mutation-associated alterations in the proteolytic processing of PS2 (Kim *et al.*, 1997b). In this system, a Triton-soluble 25 kDa C-terminal fragment (CTF) believed to represent the normal transgene-derived cleavage product, as well as a smaller Triton-insoluble 20 kDa alternative CTF were detected. Corresponding normal and alternative N-terminal fragments of 30 and 35 kDa, respectively, were also detected. In cells expressing PS2 containing the N141I Volga German FAD mutation the ratio of alternative to normal PS2 cleavage fragments was increased relative to cells expressing wild type PS2. In addition, during apoptosis endogenous PS2 was shown to be cleaved at the alternative site by a caspase-3 family protease. The caspase 3-type cleavage of PS2 was also observed in transiently or stably transfected hamster kidney cells and mouse and human neuroblastoma cells, although in these cells the N141I Volga German mutation did not have a detectable effect on processing (Loetscher *et al.*, 1997). In addition, the overexpression of a truncated form of PS2 in a mouse T cell hybridoma study was found to lead to the activation of ICE/Ced-3 cysteine proteases. The caspase 3-family proteases are CPP32-like cysteine proteases. The association of this family of molecules with the processing of PS2 is particularly intriguing in light of a number of studies which indicate that there is an association between the presenilins and apoptosis, a subject that is dealt with in detail elsewhere in this volume (Wolozin, 1998).

Both the six or eight transmembrane domain topology described earlier dictate that the N- and C-terminal domains and the site for PS2 proteolytic cleave are on the cytoplasmic side of the membrane (see Figure 1). Accordingly, both models dictate that the enzymes responsible for the proteolytic cleavage of PS2, at either the primary cleavage site or the caspase-3 family cleavage site, would be cytoplasmic in origin (see Figure 1). The hydrophilic loop located between TMD1/2 is unique in that it is the only sizable domain present in the molecule that would be predicted to orientate towards the luminal side of the ER or Golgi.

Full length presenilin proteins are rarely detected in cells or tissues without the benefit of overexpression. Surprisingly, such overexpression does not appear to result in a corresponding increase in the amount of proteolytic fragments produced, indicating that the processing event is highly regulated. A study designed to assess possible alternative pathways for the degradation of PS2 indicate that the poly-ubiquitination of the protein in a pre-Golgi compartment leads to subsequent degradation within the proteasome path-way. This study determined that while treatment with a set of known cell-permeable protease inhibitors (pepstatin A, Pefabloc CS, E-64, leupeptin and aprotinin) had no effect on the turnover of the PS2 fragments, treatment with proteasome inhibitors (ALLN and lactacystin) dramatically increased the levels of the high molecular weight, ubiquitinated forms of PS2.

Notably, the exact size of the PS2 cleavage fragments appears to vary with the cell type examined. For example, cleavage in human H4 neuroglioma cells appears to produces N- and C-terminal fragments (NTF and CTF) of approximately 30 and 25 kDa respectively, while in monkey COS cells the sizes have been reported to be a 35–40 kDa NTF and a 19 kDa CTF and in mouse neuroblastoma N2a cells the NTF has been reported at 35 kDa and the CTF at 23 kDa. As described above, the tetracycline-regulated overexpression of wild type or of the N141I mutant form of PS2 in human H4 neuroglioma leads to an increase of caspase-3 like cleavage at a site C-terminal to the site that is normally used in these cells. A detailed and extensive analysis of normal and alternative PS2 cleavage

fragments in a variety of cell types will be necessary to determine whether the observed variations in the processing of PS2 is dependent on species or cell type, on the levels of expression of the protein, on the presence of the FAD-associated mutations, and/or on the methods used for exogenous production of PS2.

Consistent with the a primary localization in the intracellular membranes of the ER and Golgi is the finding that PS2 is not post-translationally sulfated or glycosylated (Kovacs *et al.*, 1996; Walter *et al.*, 1996). PS2 does appear to be post translationally phosphorylated on serine residues located in the N-terminal of the molecule, at serines 7, 9 and 19 (Walter *et al.*, 1996). The study found that the phosphorylation of PS2 was not altered by either activation or inhibition of protein kinase C indicating that PKC is not involved in the process. The region of PS2 which contains the phosphorylation site is a particularly acidic domain which is one of the few that is not conserved between PS1 and PS2. The consequences of this lack of homology appear to be that PS1 does not serve as a substrate for N-terminal serine phosphorylation, although PS1 is phosphorylated at other sites within the molecule (Walter *et al.*, 1996). Whether the contrasting phosphorylation patterns of the N-termini of the presenilins is related to differing functions of the two molecules is not clear. At first examination, the phosphorylation state of PS2 does not appear to alter the overall pattern of localization of the molecule (Walter *et al.*, 1997), however clues to whether phosphorylation has subtle effects on subcellular localization or on the processing or function of PS2 await a further examination. A detailed analysis of presenilin phosphorylation can be found elsewhere in this volume (Walter and Haass, 1998).

PRESENILIN 2 AND A β FORMATION

One of the hallmarks of Alzheimer's disease is the deposition of amyloid beta (A β) in the form of senile plaques in the brains of patients affected with the disease. A β is a 39–42 amino acid peptide that is derived from a larger amyloid- β precursor protein (APP) which is a ubiquitously expressed type 1 integral transmembrane protein. Rare autosomal dominant mutations in APP that lie within or very close to the A β domain result in alterations in the amount and types of A β produced as well as the early-onset of AD. The N141I FAD mutation in PS2 and other FAD defects in PS1 and PS2 lead to increased production of amyloid β 42 in fibroblasts and in plasma (Scheuner *et al.*, 1996) of FAD patients as well as in transfected cells (Tomita *et al.*, 1997). Mutations in PS1 have similar effects on the generation of A β (for review, see Thinakaran and Sisodia, this volume). Currently, it is unclear whether the mutations in the presenilin proteins alter the production of A β via a direct interaction with APP or by alterations of interactions with other proteins in the A β production pathway (see [Figure 2](#)). A number of observations led our group and others to investigate the possibility of a direct interaction between PS2 and APP. The first it that the independent mutation analyses of two *C. elegans* proteins which share significant homology with the presenilins (*sel-12* and *spe-4*), have led to the hypotheses that both of these genes play a role in the intracellular trafficking and/or recycling of proteins. Likewise, it appears likely that the cellular events which lead to the production of A β and amyloid include alterations in the intracellular trafficking, recycling and processing of APP. Finally, specific populations of APP and of both of the presenilins can both be found within the same subset of intracellular ER and Golgi membranes, and a number of recent reports suggest that in neurons the site of intracellular A β generation is the ER (Chyung *et al.*, 1997; Hartmann *et al.*, 1997). In a recent study

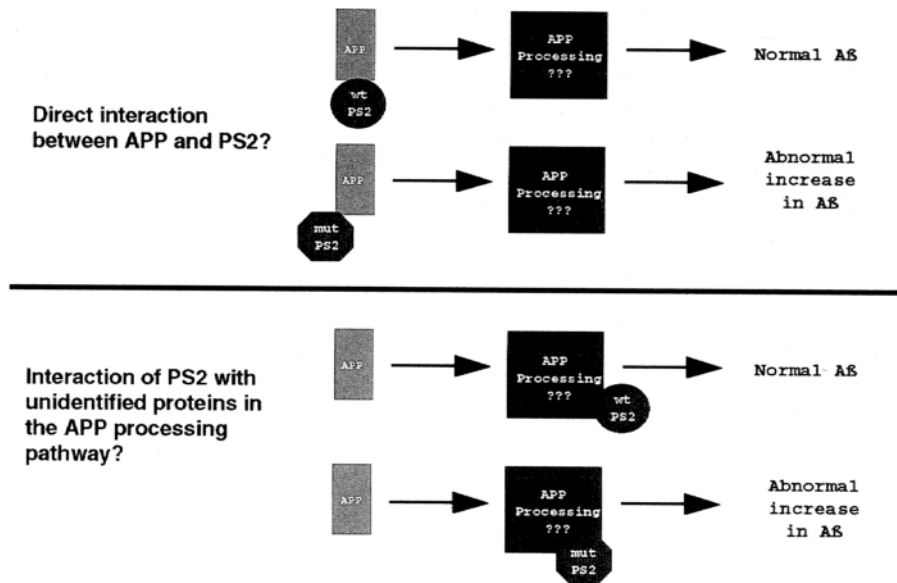


Figure 2. Direct or Indirect Interactions of PS2 and APP? It is clear that mutations in PS2 affect the amounts and types of Aβ that are produced. Whether these changes are due to mutation-induced alterations of a direct interaction with APP (top) or by alterations of interactions with other, as of yet unidentified proteins in the Aβ production pathway (bottom) remains unclear.

by Weideman *et al.* (1997) radiolabelled proteins derived from PS2 transfected COS cells were used to demonstrate that a fraction of APP was associated with PS2 immunocomplexes. Interestingly, it was the immature, N-glycosylated forms of APP and not the mature N- and O-glycosylated forms that were found to co-immunoprecipitate with PS2. Similar results were presented by Xia *et al.* (1997) who also reported that immature N-glycosylated forms of APP were able to co-immunoprecipitate with both mutant and wild type PS1 or PS2. This study also found that treatment with brefeldin A or incubation at 20° did not affect the interaction, suggesting that the complex was formed in the ER. Our group has also found that PS2 and APP co-immunoprecipitate, and through the use of membrane permeable cross-linking reagent, we have confirmed and extended these findings *in vivo* (Wasco *et al.*, 1998). The results of our studies indicate that the N141I mutation in PS2 does not alter the ability of the protein to bind APP when both proteins are removed from the lipid bilayer for co-immunoprecipitation analysis. However, when the proteins are crosslinked *in vivo* prior to treatment with detergent, the ability of N141I mutant PS2 to interact with APP is reduced as compared to wild type PS2. These results are consistent with the hypothesis that a mutation-induced conformational change in PS2 alters the ability of the molecule to interact with APP. The finding that there is a detectable interaction between PS2 and APP suggests that PS2 is directly involved in the intracellular processing and/or trafficking of APP in the ER and that mutation induced alterations in the interactions of PS2 and APP may be responsible for the observed FAD-associated increases in intracellular Aβ generation that take place within this compartment.

CONCLUSION

Although it is clear that specific mutations in PS2 lead to the production of a protein that is associated with the early-onset of FAD as well as with relative increases in the production of A β 42, the mechanism by which these mutations operate remains unknown. Current data indicate that normally, full length PS2 is present in sparingly small amounts in the cell, that it is processed by at least two different classes of proteases and that elevations in its production result in the initiation of the apoptotic cascade. A clearer understanding of the events that regulate the pro-apoptotic property of PS2 may provide information that will be useful to address the possibility that mutation-induced alterations in this function are involved in the etiology of FAD. In addition, the continued assessment of direct or indirect interactions between PS2 and APP should provide clues about how the disease-associated mutations in both of these proteins lead to the abnormal cellular trafficking/processing of APP and ultimately to the generation of A β 42.

REFERENCES

- Bird, T.D., Levy-Lehad, E., Poorkaj, P., Shwama, V., Nemens, E., Lahad, A., Lampe, T.H. and Schellenberg, G.D. (1996) Wide range in age of onset form chromosome 1-related familial Alzheimer's disease. *Ann. Neurol.*, **40**, 932–936.
- Benovic, S.A., McGowan, E.M., Rothwell, N.J., Mutton, M., Morgan, D.G., and Gordon, M.N. (1997) Regional and cellular localization of presenilin-2 RNA in rat and human brain. *Exp. Neurol.*, **145**, 555–564.
- Berezovska, O., Xia, M.Q., Page, K., Wasco, W., Tanzi, R.E., and Hyman, B.T. (1997) Developmental regulation of presenilin mRNA expression parallels notch expression. *J. Neuropathol. Exp. Neurol.*, **56**, 40–44.
- Blanchard, V., Cxeck, C., Bonci, B., Clavel, N., Gohin, M., Dalet, K., Revah, E., Pradier, L., Imperato, A., and Moussaoui, S. (1997) Immunohistochemical analysis of presenilin 2 expression in the mouse brain: distribution pattern and co-localization with presenilin 1 protein. *Brain Res.*, **758**, 209–217.
- Boissiere, E., Pradier, L., Delaere, P., Faucheux, B., Revah, F., Brice, A., Agid, Y., and Hirsch, E.C. (1996) Regional and cellular presenilin 2 (STM2) gene expression in the human brain. *Neuroreport*, **7**, 2021–2025.
- 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 1–42/1–40 ratio *in vitro* and *in vivo*. *Neuron*, **17**, 1005–1013.
- Boteva, K., Vitek, M., Mitsuda, H., Har de Silva, P.T., Xu, G., Small, G., and Gilbert, J.R. (1996) Mutation analysis of presenilin 1 gene in Alzheimer's disease. *Lancet*, **347**, 130–131.
- Cook, D.G., Sung, J.C., Golde, T.E., Felsenstein, K.M., Wojczk, B.S., Tanzi, R.E., Trojanowski, J.Q., V.M.-Y. Lee, and Doms, R.W. (1996) Expression and analysis of presenilin 1 in a human neuronal system: Localization in cell bodies and dendrites. *Proc. Natl. Acad. Sci. USA*, **93**, 9223–9228.
- Crook, R., Ellis, R., Shanks, M., Thal, L.J., Perez-Tur, J., Baker, M., Hutton, M., Haltia, T., Hardy, J., and Galasko, D. (1997) Early-onset Alzheimer's disease with a presenilin-1 mutation at the site corresponding to the Volga German presenilin-2 mutation. *Ann. Neurol.*, **42**, 124–128.

- Chyung, A.S.C., Greenberg, B.D., Cook, D.G., Doms, R.W., and Lee, V.M. (1997) Novel β -secretase cleavage of beta-amyloid precursor protein in the endoplasmicreticulum/intermediate compartment of NT2N cells. *J. Cell Biol.*, **138**, 671–680.
- Deng, G., Pike, C.J., and Cotman, C.W. (1996) Alzheimer-associated presenilin-2 confers increased sensitivity to apoptosis in PC 12 cells. *FEBS Lett.*, **397**, 50–54.
- De Strooper, B., Beullens, M., Contrera, S.B., Levesque, L., Craessaerts, K., Cordell, B., Moechars, D., Bollen, M., Fraser, P., George-Hyslop, P.S., and Van Leuven, F. (1997) Phosphorylation, subcellular localization, and membrane orientation of the Alzheimer's disease-associated presenilins. *J. Biol. Chem.*, **272**, 3590–3598.
- Doan, A., Thinakaran, G., Borchelt, D.R., Slunt, H.H., Ratovitsky, T., Podlisny, M., Selkoe, D.J., Seeger, M., Gandy, S.E., Price, D.L., and Sisodia, S.S. (1996) Protein topology of presenilin 1. *Neuron*, **17**, 1023–1030.
- Dewji, N.N., and Singer, S.J. (1997) Cell surface expression of the Alzheimer's disease-related presenilin proteins. *Proc. Natl. Acad. Sci. USA*, **94**, 9926–9931.
- 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 β 40/42 amyloid peptides. *Nat. Med.*, **3**, 1016–1020.
- Hutton, M., Busfield, F., Wragg, M., Crook, R., Perez-Tur, J., Clark, R.F., Prihar, G., Talbat, C., Phillips, H., Wright, K., Baker, M., Lendon, C., Duff, K., Martinez, A., Houlden, H., Nichols, A., Karran, E., Roberts, G., Venter, J.C., Adams, M.D., Cline, R.T., Phillips, C.A., Fuldner, R.A., Hardy, J., and Goate, A. (1996) Complete analysis of the presenilin 1 gene in families with earlyonset Alzheimer's disease. *Neuroreport*, **7**, 801–805.
- Hong, C.S., and Koo, E.H. (1997) Isolation and characterization of Drosophila presenilin homolog, *Neuroreport*, **8**, 665–668.
- Kim, T.-W., PettingeU, W.H., Hallmark, O.G., Moir, R.D., Wasco, W., and Tanzi, R.E. (1997a) Endoproteolytic cleavage and proteasomal degradation of presenilin 2 in transfected cells. *J. Biol. Chem.*, **272**, 11006–11010.
- Kim, T.W., PettingeU, W.H., Jung, Y.K., Kovacs, D.M., and Tanzi, R.E. (1997b) Alternative cleavage of Alzheimer-associated presenilins during apoptosis by a caspase-3 family protease. *Science*, **277**, 373–376.
- Kovacs, D.M., Fausett, H.J., Page, K.J., Kim, T-W, Mori, R.D., Merriam, D.E., Hoillister, R.D., Hallmark, O.G., Mancini, R., Felsenstein, K.M., Hyman, B.T., Tanzi, R.E., and Wasco, W. (1996) Alzheimer associated presenilins 1 and 2: Neuronal expression in brain and localization to intracellular membranes in mammalian cells. *Nature Medicine*, **2**, 224–229.
- Lah, J.J., Heilman, C.J., Nash, N.R., Rees, H.D., Yi, H., Counts, S.E., and Levey, A.I. (1997) Light and electron microscopic localization of presenilin-1 in primate brain. *J. Neurosci.*, **17**, 1971–1980.
- Lee, M.K., Slunt, H.H., Martin, L.J., Thinakaran, G., Kim, G., Gandy, S.E., Seeger, M., Koo, E., Price, D.L., and Sisodia, S.S. (1996) Expression of presenilin 1 and 2 (PS1 and PS2) in human and murine tissues. *J. Neurosci.*, **16**, 7513–7525.
- Lehmann, S., Chiesa, R., and Harris, D.A. (1997) Evidence for a six-transmembrane domain structure of presenilin 1. *J. Biol. Chem.*, **272**, 12047–12051.
- Levitan, D., and Greenwald, I. (1995) Facilitation of lin-12-mediated signalling by sel-12, a Caenorhabditis elegans S182 Alzheimer's disease gene. *Nature*, **377**, 351–354.
- Levitan, D., Doyle, T.G., Brousseau, D., Lee, M.K., Thinakaran, G., Slunt, H.H., Sisodia, S.S., and Greenwald, I. (1996) Assessment of normal and mutant human presenilin function in Caenorhabditis elegans. *Proc. Natl. Acad. Sci. USA*, **93**, 14940–14944.

- Levy-Lahad, E.*, Wasco, W.*, Poorkaj, P., Romano, D.M., Oshima, J.M., Pettingell, W.H., Yu, C., Jondro, P.D., Schmidt, S.D., Wang, K., Crowley, A.C., Fu, Y.-H., Guenette, S.Y., Galas, D., Nemens, E., Wijsman, E.M., Bird, T.D., Schellenberg, G.D., and Tanzi, R.E. (1995) Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science*, **269**, 973–977. (*shared first author)
- Levy-Lehad, E., Poorkaj, P., Wang, K., Fu, Y.H., Oshima, J., Mulligan, J., and Schellenberg, G.G. (1996) Genomic structure and expression of STM2, the chromosome 1 familial Alzheimer disease gene. *Genomics*, **34**, 196–204.
- Li, J., Ma, J., and Potter, H. (1995) Identification and expression analysis of a potential familial Alzheimer disease gene on chromosome 1 related to AD3. *Proc. Natl. Acad. Sci. USA*, **92**, 12180–12184.
- Li, J., Xu, M., Zhou, H., Ma, J., and Potter, H. (1997) Alzheimer presenilins in the nuclear membrane, interphase kinetochores, and centrosomes suggest a role in chromosome segregation. *Cell*, **90**, 917–927.
- Li, X., and Greenwald, I. (1996) Membrane topology of *C. elegans* SEL-12 protein. *Neuron*, **17**, 1015–1021.
- L'Hernault, S.W., and Arduengo, P.M. (1992) Mutation of a putative sperm membrane protein in *Caenorhabditis elegans* prevents sperm differentiation but not its associated meiotic divisions. *J. Cell Biol.*, **119**, 55–68.
- McMillan, P.J., Leverenz, J.B., Poorkaj, P., Schellenberg, G.D., and Dorsa, D.M. (1996) Neuronal expression of STM2 mRNA in human brain is reduced in Alzheimer's disease. *J. Histochem. Cytochem.*, **44**, 1215–1222.
- Mitsuda, T., Roses, A.D. and Vitek, M.P. (1997) Transcriptional regulation of the mouse presenilin-1 gene. *J. Biol. Chem.*, **272**, 23489–23497.
- Perez-Tur, J., Froelich, S., Prihar, G., Crook, R., Baker, M., Duff, K., Wragg, M., Busfield, F., Lendon, C., Clark, R.F., Roques, P., Fuldner, R.A., Johnston, J., Cowburn, R., Forsell, C., Axelman, K., Lilius, L., Houlden, H., Karran, E., Roberts, G.W., Rossor, M., Adams, M.D., Hardy, J., Goate, A., Lannfelt, L., and Hutton, M. (1995) A mutation in Alzheimer's disease destroying a splice acceptor site in the presenilin-1 gene. *Neuroreport*, **7**, 297–301.
- Prihar, G., Fuldner, R.A., Perez-Tur, J., Lincoln, S., Duff, K., Crook, R., Hardy, J., Philips, C.A., Venter, C., Talbot, C., Clark, R.F., Goate, A., Li, J., Potter, H., Karran, E., Roberts, G.W., Hutton, M., and Adams, M.D. (1996) Structure and alternative splicing of the Presenilin-2 gene. *Neuroreport*, **7**, 1680–1684.
- Rogaev, E.I., Sherrington, R., Rogaeva, E.A., Levesque, G., Ikeda, M., Liang, Y., Chi, H., Lin, C., Holman, K., Tsuda, T., Mar, L., Sorbi, S., Nacmias, B., Piacentini, S., Amaducci, L., Chumakov, I., Cohen, D., Lannfelt, L., Fraser, P.E., Rommens, J.M., and St George-Hyslop, P.H. (1995) Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. *Nature*, **376**, 775–778.
- Rogaev, E.I., Sherrington, R., Wu, C., Levesque, G., Liang, Y., Rogaeva, E.A., Ikeda, M., Holman, K., Lin, C., Lukiw, W.J., de Long, P.J., Fraser, P.E., Rommens, J.M., and St George-Hyslop, P. (1997) Analysis of the 5' Sequence, Genomic Structure, and Alternative Splicing of the presenilin-1 Gene (PSEN1) Associated with Early Onset Alzheimer Disease. *Genomics*, **40**, 415–424.
- Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T.D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-Lahad, E., Viitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R.E., Wasco, W., Lannfelt, L., Selkoe, D., and Younkin, S. (1996) A β 42(43) is increased *in vivo* by the PS 1/2 and APP mutations linked to familial Alzheimer's disease. *Nature Medicine*, **2**, 865–870.
- Sherrington, R., Rogaev, E.I., Liang, Y., Rogaeva, E.A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., Tsuda, T., Mar, L., Foncin, J.-F., Bruni, A.C., Montesi, M.P., Sorbi, S., Rainero, L., Pinessi, L., Nee, L., Chumakov, I., Pollen, D., Brookes, A., Sanseau, P., Polinsky, R.J., Wasco, W., Da Silva, H.A.R., Haines, J.L., Pericak-Vance, M.A., Tanzi, R.E., Roses, A.D., Fraser, P.E., Rommens, J.M., and St George-Hyslop, P.H. (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature*, **375**, 754–760.

- Takami, K., Terai, K., Matsuo, A., Walker, D.G., and McGeer, P. (1997) Expression of presenilin-1 and -2 mRNAs in rat and Alzheimer's disease brains. *Brain Res.*, **748**, 122–130.
- Tanzi, E.E., Kovacs, D.D.M., Kim, T.-W., Moir, P.D., Guenette, S.Y., and Wasco, W. (1996) *Neurobiol. Dis.*, **3**, 159–168.
- Thinakaran, G., Borchelt, D.R., Lee, M.K., Slunt, H.H., Spitzer, L., Kim, G., Ratovitski, T., Davenport, F., Nordstedt, C., Seeger, M., Hardy, J., Levey, A.I., Gandy, S.E., Jenkins, N., Copeland, N., Price, D.L., and Sisodia, S.S. (1996) Endoproteolysis of presenilin 1 and accumulation of processed derivatives *in vivo*. *Neuron*, **17**, 181–190.
- Tomita, T., Maruyama, K., Saido, T.C., Kume, H., Shinozaki, K., Tokuhira, S., Capell, A., Walter, J., Grunberg, J., Haass, C., Iwatsubo, T., and Obata, K. (1997) The presenilin 2 mutation (N141I) linked to familial Alzheimer disease (Volga German families) increases the secretion of amyloid β protein ending at the 42nd (or 43rd) residue. *Proc. Natl. Acad. Sci. USA*, **94**, 2025–2030.
- Tsujimura, A., Yasojima, K., and Hashimoto-Gotoh, T. (1997) Cloning of *Xenopus* presenilin- α and - β cDNAs and their differential expression in oogenesis and embryogenesis. *Biochem. Biophys. Res. Commun.*, **231**, 392–396.
- Vito, P., Lancana, E., and D'Adamio, L. (1996) Interfering with apoptosis: Ca(2+)-binding protein ALG-2 and Alzheimer's disease gene ALG-3. *Science*, **271**, 521–525.
- Vito, P., Wolozin, B., Ganjei, J.K., Iwasaki, K., Lacana, E., and D'Adamio, L. (1996) Requirement of the familial Alzheimer's disease gene PS2 for apoptosis. Opposing effect of ALG-3. *J. Biol. Chem.*, **271**, 31025–31028.
- Walter, J., Capell, A., Grunberg, J., Pescold, B., Schindzielorz, A., Prior, R., Podlisny, M.B., Fraser, P., St. George-Hyslop, P., Selkoe, D.J. and Haass, C. (1996) The Alzheimer's disease-associated presenilins are differentially phosphorylated proteins located predominantly within the endoplasmic reticulum. *Molec. Med.*, **2**, 673–691, 19964–5111.
- Walter, J. and Haass, C. (1998) The phosphorylation of presenilin proteins, this volume.
- Wasco, R.E., Tanzi, R.D., Moir, A.C., Crowley, D.E., Merriam, D.M., Romano, P.D., Jondro, and Kellerman, B.A., Presenilin 2—APP Interactions. Presenilins and Alzheimer's Disease. Eds. R.E. Tanzi, S. Younkin and Y. Christen, Springer-Verlag Press, pp. 59–70.
- Weidemann, A., Paliga, K., Durrwang, U., Czech, C., Evin, G., Masters, C.L., and Beyreuther, K. (1997) Formation of stable complexes between two Alzheimer's disease gene products: presenilin-2 and β -amyloid precursor protein. *Nat. Med.*, **3**, 328–332.
- Wolozin, B., Iwasaki, K., Vito, P., Ganjei, J.K., Lacana, E., Sunderland, T., Zhao, B., Kusiak, J.W., Wasco, W., and D'Adamio, L. (1996) Participation of presenilin 2 in apoptosis: enhanced basal activity conferred by an Alzheimer mutation. *Science*, **274**, 1710–1713.
- Wolozin, B. (1998) Presenilin proteins and their role in apoptosis, this volume.
- Xia W, Zhang, J., Perez, R., Koo, E.H., and Selkoe, D.J. (1997b) Interaction between amyloid precursor protein and presenilins in mammalian cells: Implications for the pathogenesis of Alzheimer's disease. *Proc. Natl. Acad. Sci. USA*, **9**, 8208–8213.

12. PRESENILIN PROTEINS AND THEIR ROLE IN DEVELOPMENT AND NOTCH SIGNALING

RALF BAUMEISTER¹ AND CHRISTIAN HAASS²

¹*LMB/Genzentrum der LMU München, Feodor-Lynen-Str. 25, D-81377 München,
Germany, and*

²*Zentralinstitut für Seelische Gesundheit, J5, D-68159 Mannheim, Germany*

The presenilins (PS) are a family of transmembrane proteins that are widespread and share a high cross-species conservation. Presenilin genes have so far been isolated from human, mouse, rat, *Xenopus*, *Drosophila* and the nematode *Caenorhabditis elegans*. They all share significant similarities of both primary sequence and structure (Figure 1). From hydropathy plots of their amino acid composition a seven to ten transmembrane domain model had been suggested (Sherrington *et al.*, 1995). This model was subsequently confirmed in topology studies of human and *C. elegans* presenilins (Doan *et al.*, 1996; Li *et al.*, 1996). The broad distribution in the animal kingdom and the high degree of structural conservation suggest that presenilins may have similar functions.

In this review, we summarize our current knowledge about presenilin function obtained in the various model systems and how this relates to the role of human presenilins in Alzheimer's disease. In the first part we will compare the expression patterns of presenilin proteins during the development of the respective organisms. The spatio-temporal expression of the presenilins, together with the lethal phenotype of PS1 knock-out mice, does not correlate with the onset of Alzheimer's disease in humans and strongly suggests that the presenilins have additional functions prior to their pathogenic contribution to the disease. In the second part we will discuss the phenotypic consequences of presenilin mutations in *C. elegans* and mouse, as well as conclusions drawn from overexpressing presenilin variants in various models. In this context, we will primarily focus on their role in the Notch signaling pathway. The involvement of presenilins in apoptosis will be discussed in the following chapter.

EXPRESSION PATTERNS OF PRESENILINS IN VARIOUS ORGANISMS SUGGEST A ROLE IN EMBRYOGENESIS AND CELLULAR DIFFERENTIATION

Mammalian Presenilin Genes PS-1 and PS-2

The two human presenilin genes were cloned (Sherrington *et al.*, 1995; Levy-Lahad *et al.*, 1995b; Rogaev *et al.*, 1995) based on the contribution of their mutations to an early onset of familial Alzheimer's disease (FAD). They were mapped to chromosomes 14q24.3 (PS1: initially labeled

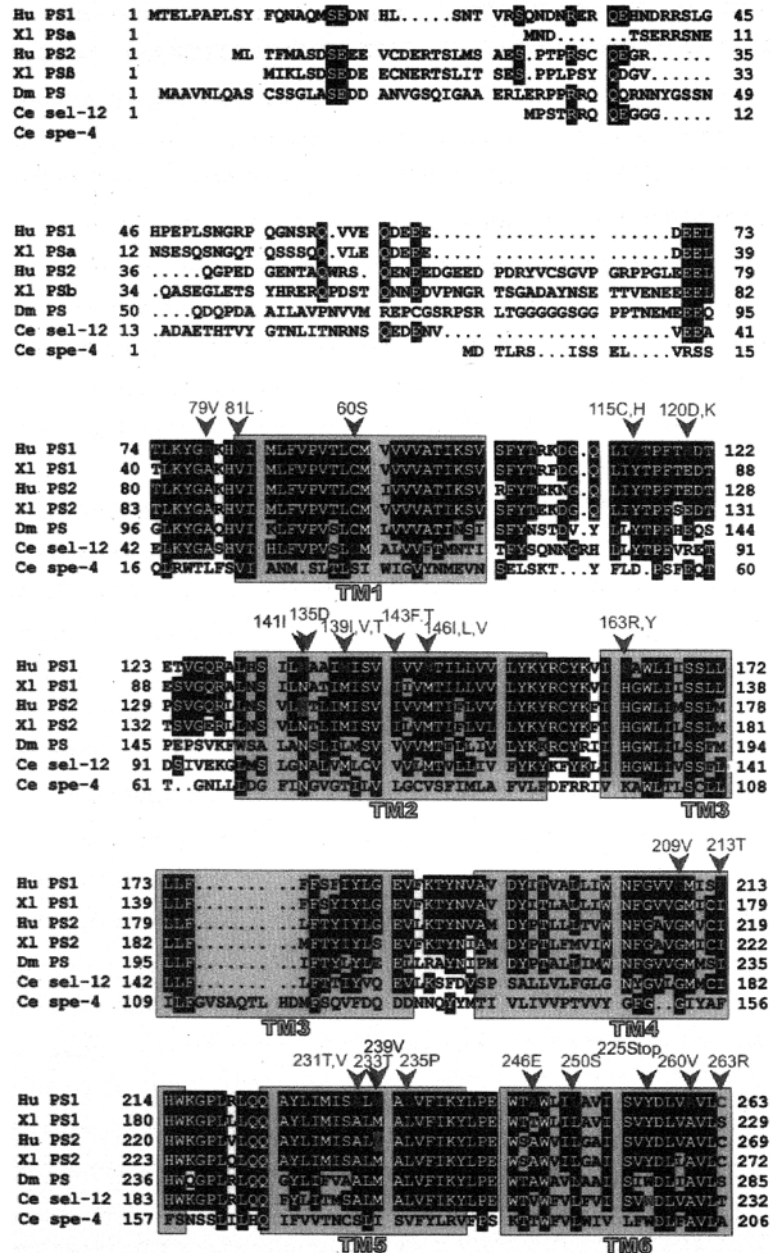
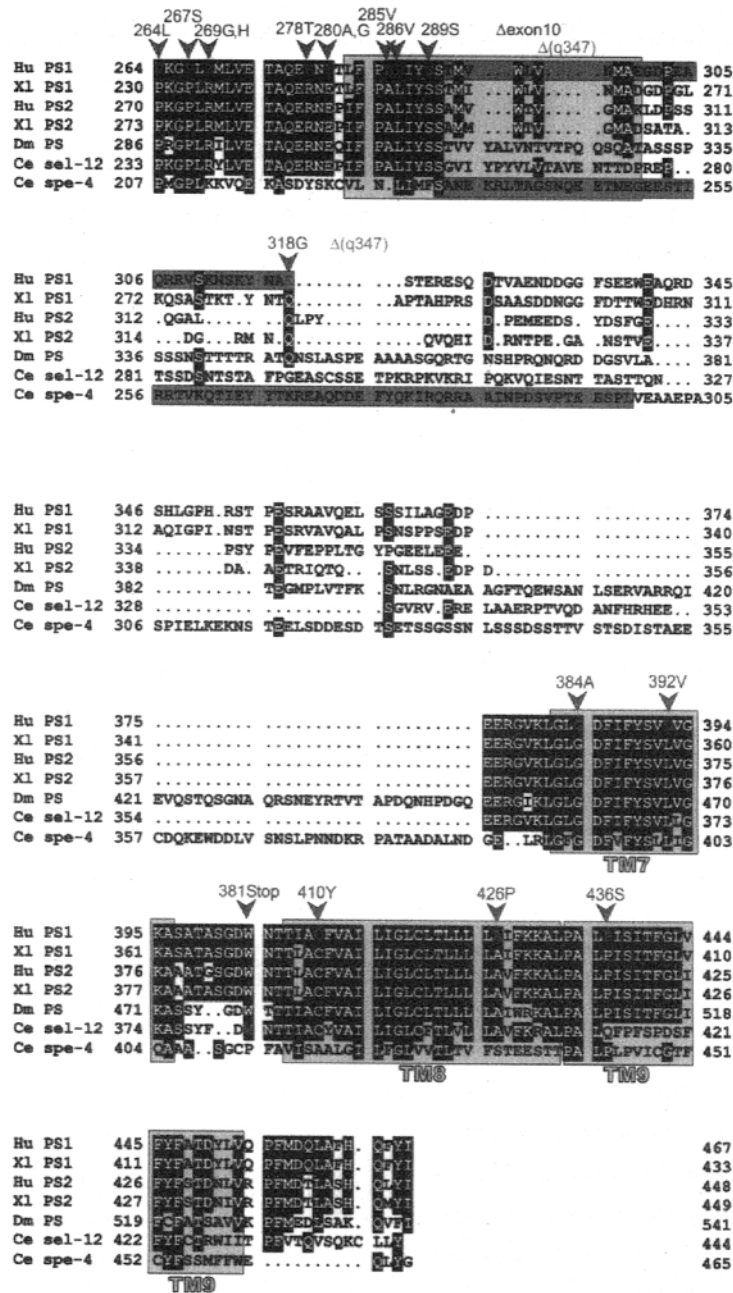


Figure 1. Presenilin sequence alignment. Amino acid sequences of presenilins from mammals (represented by human PS1 and PS2), *Xenopus laevis* (X-PS- α and X-PS- β), *C. elegans* (SEL-12 and SPE-4), and *Drosophila melanogaster* (DPS) are aligned, with abbreviated names indicated to the left. Amino acids that are conserved in at least four proteins are drawn in white on black background. Yellow boxes indicate predicted transmembrane (TM) domains (as suggested by Hardy, 1997a). The residues mutated in FAD linked and *C. elegans* presenilins are indicated, together with the



replacing residue. Red: PS1 mutations and Δexon10 deletion, blue: PS2 mutations, green: *sel-12* mutations; magenta: *spe-4* deletion. The data for these figures are mostly taken from de Silva and Patel (1997) and from Hardy (1997a,b). (See Colour Plate II)

S182) and Iq42.1 (Takano *et al.*, 1997) (PS2: initially labeled STM2) and encode proteins of 467 and 448 amino acids, respectively, which are 67% identical in primary sequence. They are highly similar

to the presenilins of rat and mouse. The 468 amino acid rat presenilin-1 protein has 88% and 93% amino acid similarity with those of human PS1 and mouse PS1, respectively (Takahashi *et al.*, 1996).

The human PS genes are expressed in the brain and in several peripheral tissues (Sherrington *et al.*, 1995; Levy-Lahad *et al.*, 1995a). Mouse PS1 is broadly expressed in a variety of tissues, including the embryonic and adult brain (Elder *et al.*, 1996; Moussaoui *et al.*, 1996; Shen *et al.*, 1997; Wong *et al.*, 1997). Expression levels are significantly higher during the development of the mouse brain than in the brain of adult mice. Expression is particularly abundant in the ventricular zone of the developing brain, where Notch is also expressed abundantly (see below for discussion). For a detailed description of the mouse PS1 expression pattern, see Shen *et al.* (1997) and Wong *et al.* (1997). Rat PS1 mRNA was detected predominantly in neurons. It is most abundant in hippocampal formation and cerebellar granule cell layers. No or only very low expression could be detected in the glial cells of the white matter, in fibrous astrocytes and oligodendrocytes (Kovacs *et al.*, 1996; Quarneron *et al.*, 1996). Similarly, PS1 is also expressed throughout the adult life of rodents and humans.

Like PS1, PS2 is also expressed predominantly in the brain of mouse, rat and human (Blanchard *et al.*, 1997; Benkovic *et al.*, 1997).

C. elegans* Presenilin Genes *sel-12*, *spe-4* and *cps-3

The first non-human presenilin genes were isolated from the nematode *Caenorhabditis elegans*. Both *C. elegans* presenilin genes *spe-4* and *sel-12* genes were identified in genetic screens not related to the study of Alzheimer's disease. In fact, *spe-4* had been cloned several years before the contribution of the presenilin mutants to the early onset of AD was revealed (L'Hernault and Arduengo 1992).

The *spe-4* gene encodes a 465 amino acid protein that shares 24% amino acid identity with human PS1 (Sherrington *et al.*, 1995). *spe-4* seems to be expressed exclusively in the spermatocytes. Consistent with this result, the probable null phenotype of mutants is limited to the disruption of spermatogenesis.

sel-12 was identified by reverting the phenotype of a constitutively active *lin-12*/Notch mutant (Levitan and Greenwald, 1995) functionally implicating it in Notch signaling (for a detailed discussion, see below), *sel-12* encodes a 461 amino acid protein that displays 47% amino acid sequence identity to PS1 and PS2. It was subsequently shown that *sel-12* is expressed at all developmental stages from embryo to adult (Levitan *et al.*, 1996; Baumeister *et al.*, 1997). With the exception of the intestine, expression of reporter constructs under the control of the *sel-12* promoter is seen in most, if not all, neuronal cells, and many non-neuronal cell lineages (Baumeister *et al.*, 1997). Genetic analyses established that a reduction or elimination of SEL-12 activity results in an egg-laying defect of the mutant animals. Since the vulva and egg-laying apparatus develops in late larval stages, it is unlikely that these structures are the only ones affected in *sel-12* mutants. Expression in the vulva precursor cell (VPC) lineages and the somatic gonadal cells can be associated with the egg-laying defect of *sel-12* mutants. Compromised neuronal function associated with the reduced activity of *sel-12* is much more difficult to prove, and

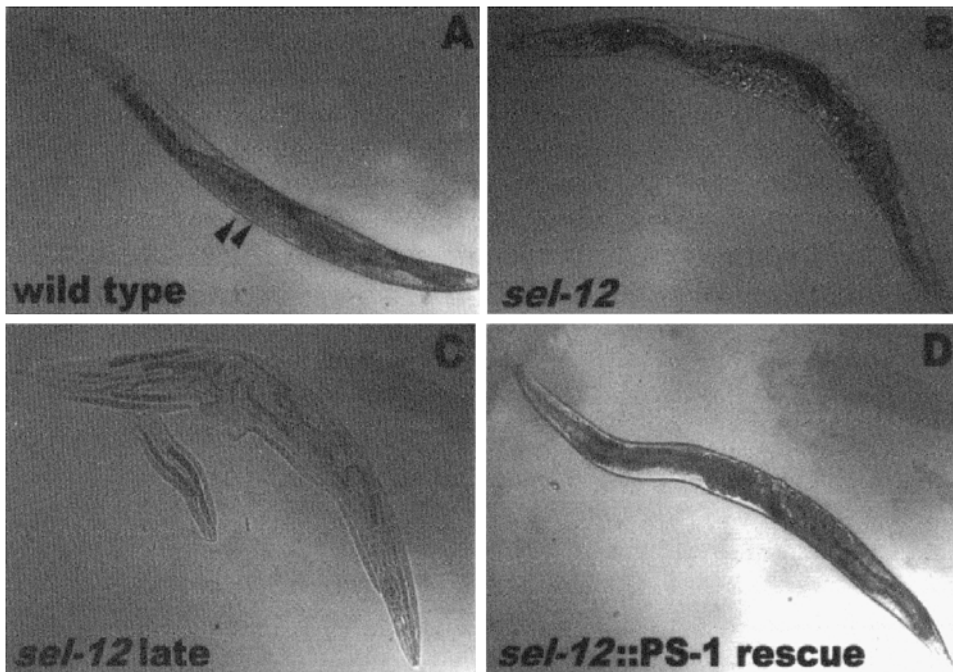


Figure 2. Human PS1 complements the egg-laying defect of *C. elegans sel-12* (ar131). (A) A *C. elegans* wild-type adult animal carries 17 ± 4 unlaidd fertilized eggs *in utero*. Arrowheads point to two of the eggs. (B) Adult *sel-12* (ar131) animal. Due to the egg-laying defect, the uterus of this animal is filled with more than 50 eggs. Embryonic development is not affected by the *sel-12* mutations, and most embryos have progressed to late stages. (C) A *sel-12* (ar131) animals on its third day after reaching adulthood. The unlaidd eggs have hatched inside the uterus, resulting in the death of the animal. (D) Transformation of a PS1 cDNA expressed from the *sel-12* promoter results in complementation of the egg-laying defect of *sel-12* (ar131) animals. The transgenic animal shown here carries only 20 fertilized eggs being at early stages of development. (Modified, with permission, from Baumeister *et al.* 1997).

is indicated by the significant lack of temperature responsiveness of the mutant animals (Wittenburg and Baumeister, unpublished results).

The functional relationship between *sel-12* and human presenilins was demonstrated by the observation that the egg-laying defect of *C. elegans sel-12* mutant animals is rescued by both PS1 and PS2 (Levitan *et al.*, 1996; Baumeister *et al.*, 1997; Baumeister, unpublished reports) (Figure 2).

Recently, a putative open reading frame (ORF) encoding a third *C. elegans* presenilin was detected during the efforts to sequence the entire *C. elegans* genome (Wilson *et al.*, 1994). Therefore, *C. elegans* currently represents the only organism from which three different presenilins are known. This putative ORF, which we tentatively name *cps-3* (for *C. elegans* presenilin 3) is particularly interesting because it is much less well conserved in the transmembrane stretches, which in other presenilins have the highest degree of sequence similarity (Figure 3). No functional or expression data are currently available for *cps-3*.

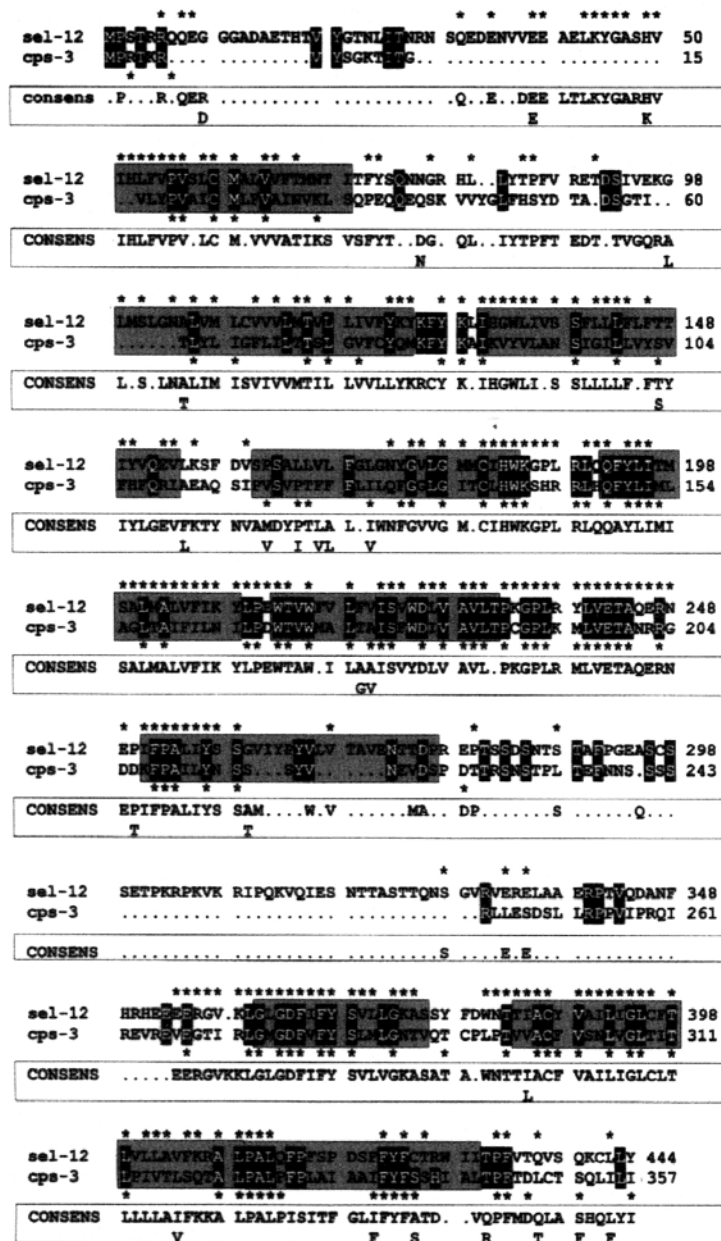


Figure 3. Third putative *C. elegans* presenilin. The Genefinder (Favella *et al.*, 1995) predicted amino acid sequence of the putative *C. elegans* presenilin CPS-3 is compared to SEL-12 and a consensus sequence derived from the alignment of presenilins in Figure 1. Sequence identity between SEL-12 and CPS-3 is indicated by white letters on black background. Transmembrane domains are shown by grey boxes. Asterisks mark amino acid identity between the consensus sequence and SEL-12 (top) or CPS-3 (bottom).

***Xenopus laevis* Presenilin Genes X-ps- α and X-ps- β**

Xenopus presenilin- α and - β cDNAs were isolated by screening an adult brain cDNA library with a human PS1 probe (Tsujimura *et al.*, 1997). Two types of cDNA clones were isolated, encoding putative proteins of 433 amino acids (X-ps- α) and 449 amino acids (X-ps- β), respectively. X-ps- α shares 89% amino acid similarity with human PS1, X-ps- β is more similar to human PS2 (with an amino acid similarity of 86%). X-ps- α and X-ps- β are ubiquitously expressed in multiple tissues, as revealed in Northern blot and competitive RT-PCR experiments. The strongest expression was detected for both genes in the ovarian tissues, whereas only weak expression was detected in skeletal muscle. Late expression was found predominantly in the CNS, with lower levels of expression elsewhere (Tsujimura *et al.*, 1997).

***Drosophila Melanogaster* DPS**

The gene for *Drosophila* presenilin (DPS) was isolated by degenerate PCR approaches and subsequently mapped to position 77BC of the third chromosome. It encodes a 541 amino acid protein (528 amino acid protein, according to Hong *et al.* (1997)) with 53% amino acid identity to human PS1 and PS2, and 45–48% to the SEL-12 protein (Boulianne *et al.*, 1997; Hong *et al.*, 1997). Whole-mount *in situ* hybridization revealed expression at multiple developmental stages. DPS was reported to be highly expressed during oogenesis in nurse cells and the developing oocyte. It later shows a dynamic expression pattern, which is consistent with it having multiple functions during *Drosophila* development. No evidence for the existence of a second presenilin was detected by Southern blot analysis. No functional data have so far been reported for DPS.

Taken together, the presenilins in the various organisms are expressed predominantly in the brain, but also in many other tissues. Expression starts in early embryogenesis and is maintained throughout the life of an animal. This predicts that the presenilins have important functions at various stages of animal life. On the other hand, there is no correlation between the expression pattern of presenilins and the susceptibility to AD pathology of cerebral structures of patients carrying the dominant, heterozygous PS1 and PS2 mutants (Benkovic *et al.*, 1997; Blanchard *et al.*, 1997). Other as yet identified cofactors might interact with mutated presenilins to cause neurodegeneration in AD affected areas.

ANALYSES OF MUTANT PHENOTYPES

The analyses of phenotypes associated with mutations in the presenilin genes are most informative for understanding their functions. All of the missense mutations (*sel-12*:C60S, *spe-4*), nonsense mutations (*sel-12*:W225stop; *sel-12*:W381stop), and deletions (*spe-4*(q347): deletion of 221 carboxyterminal amino acids) in *C. elegans*, and the null mutations isolated from mouse PS1 (Shen *et al.*, 1997; Wong *et al.*, 1997) result in a fully recessive phenotype. This is clearly in contrast to the pathogenic mutations found in early-onset FAD. More than 30 missense mutations and a single deletion of human

PS1 which segregate with the FAD phenotype are dominant (for review: Tanzi *et al.*, 1996; Selkoe 1996; Hardy 1997a,b). Since FAD mutations obviously do not cause an embryonic phenotype, we suggest that their dominant manifestation in early-onset of Alzheimer's disease is limited to their contribution in the development of this disease.

The *C. elegans* *spe-4* Presenilin Gene Functions in Protein Transport or Sorting

C. elegans spe-4 mutants are sterile. SPE-4 protein is required for the proper partitioning of cytoplasm during development of the spermatid (L'Hernault and Arduengo, 1992). This mechanism is particular for nematode sperm. It is mediated by the fibrous-body membranous organelle (FB-MO) complexes that function in the transport, prepackaging and segregation of soluble and membrane-bound proteins. FB-MO is a specialized Golgi-derived organelle, consisting of a membrane-bound vesicle attached to and partly surrounding a complex of parallel protein fibers that are predominantly formed from the major sperm protein (MSP).

SPE-4 protein seems to be localized to the FB-MO during sperm development. Ultrastructural analysis revealed that *spe-4* mutants have defects in FB-MO morphogenesis that disrupt the coordination of cytokinesis with meiotic nuclear divisions. In *spe-4* mutants, the fibrous body is not surrounded by a double membrane. As a consequence of defective *spe-4*, instead of four spermatids a spermatocyte-like cell that contains four haploid nuclei is formed and the mutant animals do not produce functional spermatozoa. This results in an arrest of sperm morphogenesis causing sterility.

The precise biochemical function of SPE-4 protein is yet unknown. It has been suggested that SPE-4 may either be required to stabilize interactions between the membrane proteins of the FB-MO during membrane fusion and budding, or be involved in the intracellular transport or segregation of the proteins of the FB-MO (L'Hernault and Arduengo, 1992).

The Role of Presenilins in Notch Signaling

As already mentioned earlier in this chapter, the second clue to the biological role of the presenilins, in addition to the function of *spe-4* in protein sorting, came from the genetic characterization of *C. elegans sel-12*. Loss-of-function alleles of *sel-12* suppress the Multivulva phenotype of constitutively active *lin-12*, a member of the Notch family of cell signaling receptors.

To understand the function of *sel-12* presenilin in *lin-12* signaling, we will now briefly review the contribution of *lin-12* to lateral signaling and differentiation in *C. elegans*. For a more detailed introduction to *lin-12*/Notch signaling, we refer to some excellent reviews on this topic (Greenwald, 1989; Artavanis-Tsakonas *et al.*, 1995).

In *C. elegans*, the *lin-12*/Notch gene is involved in several binary decisions orchestrating the development of the egg-laying apparatus. In one such decision, two descendants of the somatic gonad precursors, named Z1.ppp and Z4.aaa, display variable cell fate choices (Figure 4). One eventually becomes the anchor cell AC and the other becomes a ventral uterine precursor cell VU. AC is required

for the induction of ventral hypodermis specialization, the VU produces descendants that form the ventral uterus, *lin-12* mediates this specification and is first expressed in both uncommitted Z1.ppp and Z4.aaa (Figure 4A). After binding of its ligand LAG-2/Delta, activated LIN-12 induces VU fate in the cell autonomously. The uninduced cell becomes AC. LIN-12 activity is sufficient for VU induction, since *lin-12* null alleles which eliminate *lin-12* activity cause both Z1.ppp and Z4.aaa to become ACs (Figure 4B). In contrast, semidominant *lin-12(d)* mutations result in elevated activity of LIN-12 receptor protein, and both cells become VUs while the AC is not formed at all (Figure 4C).

Since AC is required for a series of other signaling events which are essential to induce vulva formation, the consequence of *lin-12(d)* mutation is the absence of a functional vulva and the inability of the mutant animals to lay eggs (Vulvaless phenotype). In addition, several non-functional pseudovulvae may be formed as a result of LIN-12 hyperactivity affecting other cell-cell signaling events (Multivulva phenotype).

In order to identify genes which participate in the same cell interactions that require *lin-12*, Levitan and Greenwald (1995) screened for suppressors of the *lin-12(d)* animals' Multivulva phenotype and identified *sel-12* (suppressor/enhancer of *lin-12*) (Figure 4D). All *sel-12* alleles isolated are recessive loss-of-function alleles. *sel-12* mutants can enhance the penetrance of the 2 AC phenotype of a *lin-12* hypomorphic (reduced function) allele. *sel-12* mutants can also partly suppress the penetrance of the 0 AC phenotype of a *lin-12* hypermorphic (increased function) allele. This suggests that *sel-12* mutants generally reduce *lin-12* activity and implicates SEL-12 function in the Notch signaling pathway, *sel-12* mutants also reduce the activity of *glp-1* hypomorphic alleles, another member of the Notch receptor family in *C. elegans* mediating different cell-cell interactions from the ones affected by *lin-12*. This indicates a more general role of *sel-12* in intercellular signaling in *C. elegans*.

Two groups recently reported targeted gene knock-out of mouse PS1 (Shen *et al.*, 1997, Wong *et al.*, 1997). PS1 $-/-$ mice have severe developmental defects and die postnatally, whereas no developmental deficits could be observed in PS1 $-/+$ heterozygous animals. Homozygous PS1 knock-out animals suffer from considerable defects in the formation of the vertebral column and the ribcage, as well as from intracranial hemorrhages with varying degrees of severity. The latter, however, are probably not the cause of death because (a) they rarely involve vital structures of the hindbrain and (b) their degree of severity does not correlate with the death of the animals. Death is, therefore, probably due to deformities of the ribcage impairing the respiratory mechanics of the mutants (Shen *et al.*, 1997; Wong *et al.*, 1997). The results reported also indicate that PS1 and PS2 functions are not fully redundant, because PS2 cannot substitute for PS1 activity.

Somite segmentation in PS1 $-/-$ animals is highly irregular. Using marker genes which identify specific somitic lineages, both groups report that the somite segmentation defects in PS1 $-/-$ animals are similar to those of mouse embryos with functionally inactivated Notch receptor or its ligand Delta. In fact, both Notch1 expression and Dll1 expression is greatly reduced in PS1 $-/-$ animals. This suggests that PS1 is required for the expression and function of Notch1 and Dll1 in the paraxial mesoderm. Together with the phenotype reported for *C. elegans sel-12* mutations, these results strongly support a model that the presenilins facilitate the Notch signaling pathway.

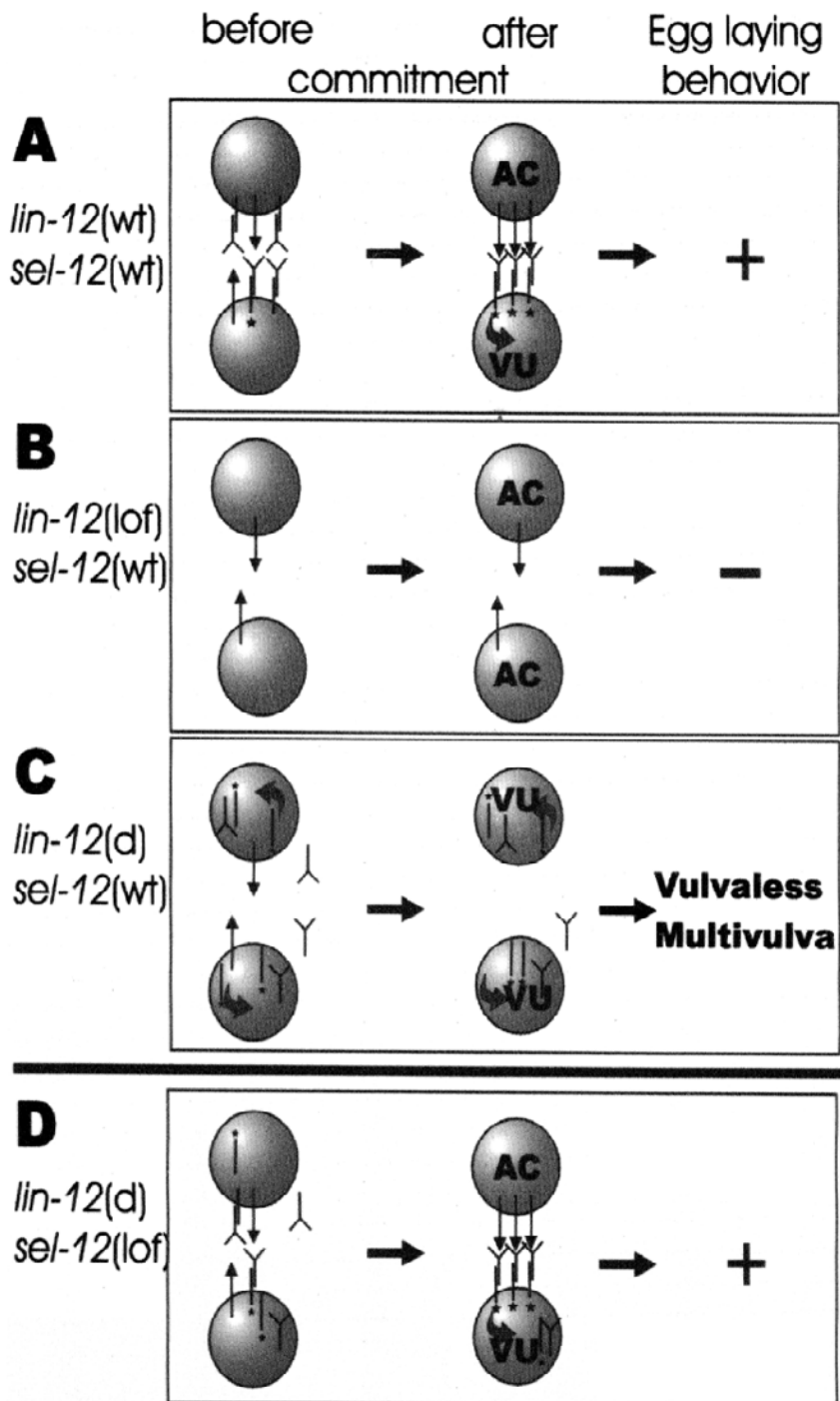


Figure 4. Proposed function of presenilin in Notch signaling. Depicted is a model for the involvement of *lin-12* in the specification of AC and VU fates during vulva formation of *C. elegans*. The Figure is adapted from a drawing in Seydoux and Greenwald (1989) and takes into account the recently proposed models for heterodimer formation of Notch by Blaumueller *et al.*, (1997) and Pan and Rubin (1997). (A) Left side: During the specification of the *C. elegans* vulva, two cells (Z1.ppp and Z4.aaa) have initially equal capacity to signal (via LAG-2/Delta; arrow) or receive (via LIN-12/Notch, open fork). In a stochastic event, signaling in one cell exceeds a critical threshold, indicated by an asterisk. This cell becomes the signaler, the other cell activates LIN-12/Notch and becomes the receiver. Right side: Activated Notch positively autoregulates *lin-12* transcription and down-regulates *lag-2* expression. Thus, the receiver is reinforced by a feedback mechanism (grey arrow) and is committed to VU fate. The signaling cell adopts AC fate. Egg-laying behavior represents the wild type (+) situation. (B) In mutants with reduced or absent LIN-12 activity (*lin-12* loss-of function (lof) or null alleles) neither cell becomes committed to be a receiver. Both cells adopt AC fate. Egg-laying behavior is defective (—). (C) In contrast, the dominant *lin-12(d)* allele n950 is hyperactive. One possible explanation for the hyperactivity of the LIN-12 point mutation A873T (Greenwald and Seydoux, 1990) is incorporated into this model. The amino acid exchange occurs in an extracellular domain of LIN-12/Notch and may prevent heterodimerization of LIN-12 N- and C-termini in the trans-Golgi network (TGN). Uncomplexed C-terminus may mimic activated LIN-12/Notch (Blaumueller *et al.*, 1997). Constitutive signaling of this mutant protein commits both cells to VU fate. Absence of AC results in the lack of a functional vulva (Vulvaless phenotype). In addition, LIN-12 hyperactivity causes a Multivulva phenotype characterized by the production of additional, non-functional ectopic pseudovulvae (Greenwald *et al.*, 1983; Ferguson and Horvitz, 1985). (D) The genetic situation of the suppressor screen by Levitan and Greenwald (1995). The Multivulva phenotype caused by LIN-12(d) hyperactivity is suppressed by loss-of-function alleles of *sel-12*. According to our model, mutant SEL-12 protein either prevents the constitutive signaling of hyperactive LIN-12(d) protein or, more likely, affects transport or retrograde recycling of LIN-12(d). It is an attractive hypothesis to assume that, in *sel-12* variants, retardation of LIN-12 mutant A873T transport through ER and Golgi might increase the probability of N- and C-terminus to heterodimerize in the TGN. Therefore, a substantial amount of active LIN-12 heterodimers might appear on the cell surface to interact with ligands. Ligand binding, in turn, would enable a quasi-normal feedback mechanism and would explain why the probability of a 1:1 ratio of AC and VU cells is raised in the double mutant. This leads to the suppression of the Multivulva phenotype which is indeed observed in the double mutant (Levitan and Greenwald, 1995). A truncated Notch construct resembling the C-terminal 100 kDa fragment is known to result in constitutive signaling, also (Kopan *et al.*, 1996). Our model suggests that the hyperactivity of the respective *lin-12* mutant would not be suppressed by *sel-12* mutations.

Genetic Determination of Presenilin Function

In summary, several lines of evidence link presenilin function to Notch signaling: (1) *sel-12* was cloned as a suppressor of hyperactive Notch mutants, thus *sel-12* wild type function facilitates signaling mediated by Notch. (2) Expression of Notch 1 and its ligand Delta1 is markedly reduced in the presomitic mesoderm of PS1 null mice. In addition, (3) the phenotype of PS1 null mice is reminiscent of the vascular changes seen in CADASIL, a clinical disease characterized by stroke and dementia, that has been linked to defects in Notch signaling (Joutel *et al.*, 1996). (4) PS1 has been shown to physically interact with members of the catenin family expressed in the brain (Zhou *et al.*, 1997). The catenins are intermediates in the wingless/wnt signaling pathway, which, like Notch, is involved in intercellular signaling. In addition, at least one protein, dishevelled, mediates interactions of both Notch and wingless pathways (Axelrod *et al.*, 1996).

What exactly is the role of the presenilins in Notch signaling? The function of *sel-12* mutants in suppressing hyperactive Notch would be consistent with both a role upstream or downstream of signaling by activated Notch, *sel-12* could function both in the signaling pathway between membrane-associated Notch and the expression control of downstream genes, as a membrane-bound component of the Notch receptor itself (facilitating ligand binding) or, at an earlier step, in the sorting and

transport of Notch from the endoplasmic reticulum to the membrane. The intracellular localization of vertebrate homologues in the membranes of the endoplasmic reticulum and Golgi strongly favor the latter model (Kovacs *et al.*, 1996). Moreover, there is evidence now that *sel-12* mutations enhance the defects of at least one other membrane-bound receptor not related to Notch (R.Baumeister, unpublished results). A more general role in protein transport and/or sorting was already suggested for the SPE-4 presenilin (L'Hernault and Arduengo 1992). PS-1 $-/-$ mice show strongly decreased expression levels of both Notch 1 and Delta 1 both of which are membrane-bound molecules. Analysis of *lin-12* Notch function in *C. elegans* (Wilkinson *et al.*, 1994) has revealed that LIN-12 activity positively autoregulates the transcription of the *lin-12* gene and represses the expression of the *lag-2*/Delta gene encoding the LIN-12 ligand. Therefore, a negative effect of mutant presenilins on LIN-12 signaling would be more consistent with elevated or unaffected expression levels of Delta than what is actually seen. Taken together, based on the experimental results which are currently available, we would propose that the presenilins have a rather general function in either the control of transport or sorting of transmembrane proteins (Figure 4). An involvement of presenilins in the quality control and retention of aberrant protein has also been suggested by others (Weidemann *et al.*, 1997). Whether the presenilins are involved in the transport of only a few or many membrane-bound proteins remains to be determined. Experiments in either *C. elegans* or PS-1 knock-out mice should give a conclusive answer within the next couple of months.

With respect to Alzheimer's disease this model suggests that the main biochemical defect of presenilin FAD mutants affects transport or sorting of the Amyloid precursor protein (APP) on its way from the ER to the cytoplasmic membrane. Several recent publications suggest a direct interaction between domains of the presenilin proteins and APP (Weidemann *et al.*, 1997, Xia *et al.*, 1997). Most importantly, Weidemann *et al.*, (1997) recently reported that this non-covalent interaction was restricted to immature forms of APP and probably occurs in the endoplasmic reticulum. This could indicate that either presenilin mutants slow down the transit of APP thus facilitating the attack of competing secretases, or they result in an incorrect sorting of APP into a compartment of the cell with different secretase levels.

Is Alzheimer's Disease Associated with a Gain-of-Misfunction Activity of Mutant Presenilins?

It has been discussed extensively whether the presenilin mutations associated with FAD are causing a loss-of-function or gain-of-misfunction activity. By genetic arguments, the *sel-12* mutations isolated by Levitan and Greenwald (1995) reduce or eliminate *sel-12* activity and, therefore, behave as loss-of-function alleles. The egg-laying phenotype of *sel-12* mutants is fully rescued by human PS1 and PS2, but not by FAD linked PS1 variants expressed from the endogenous *sel-12* promoter (Baumeister *et al.*, 1997) or from a heterologous promoter (Levitan *et al.*, 1996). In addition, the overexpression of several human FAD linked PS1 variants in wild type animals from the *sel-12* promoter (Baumeister *et al.*, 1997) did not affect the egg-laying behavior or cause a neomorphic phenotype (caused by a novel activity). It was therefore suggested that FAD mutant have a loss-of-function phenotype, at least when assayed in *C. elegans*. This is clearly in contrast to results obtained by several groups overexpressing PS1 and PS2 FAD mutant genes in transgenic animals or cultured vertebrate cells (Lemere *et al.*,

1996; Duff *et al.*, 1996, Borchelt *et al.*, 1996; Citron *et al.*, 1997). In these experiments, expression of mutant PS genes showed increased levels of A β 42 production, suggesting that the normal levels of the additional endogenous wild type protein could not compensate for the mutant. It is possible that a gain-of-misfunction phenotype of FAD mutants is limited to susceptible cell types such as neurons. Interestingly, overexpression of PS1 mutant genes from the *sel-12* promoter in *C. elegans*, although not affecting the egg-laying behavior, resulted in a significant reduction of brood size of the transgenic animals (Baumeister *et al.*, 1997). This is reminiscent of the decreased brood sizes of dominant *lin-12* alleles (Greenwald *et al.*, 1983) and may indicate that FAD mutants indeed cause a gain-of-misfunction phenotype. As a second possibility, this gain-of-misfunction activity of the presenilins is limited to their interaction with APP. Perhaps transport/mis-sorting of other membrane-bound proteins does not result in cytotoxic components like the amyloid β -peptide A β 42 which is absent from *C. elegans* (Daigle and Li, 1995). It is worth noting that all FAD linked APP mutations affecting A β 42 processing are also dominant, whereas the targeted knock-out of APP in mice does not cause any significant phenotype (Zheng *et al.*, 1995). The analysis of amyloid burden in mice heterozygous for inactivated PS1 will be most informative to answer this question.

CONCLUSION

Over the past two years since the first human presenilin was cloned, a tremendous progress has been made to understand the role of this large family of transmembrane proteins. Having now both a genetically accessible *C. elegans* model system and several mouse PS1 knockout strains available will greatly facilitate further investigation of presenilin function. The obvious functional conservation of the *C. elegans* and human presenilins, as indicated by the rescue experiments described, will hopefully allow us to extrapolate experimental results gained in each of the model organisms to enhance our understanding of presenilin (mis-) function in Alzheimer's disease.

REFERENCES

- Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M.E. (1995) Notch Signaling. *Science*, **268**, 225–232.
- Axelrod, J.D., Matsuno, K., Artavanis-Tsakonas, S. and Perrimon, N. (1996) Interaction Between Wingless and Notch Signaling Pathways Mediated by Dishevelled. *Science*, **271**, 1826–1832.
- Baumeister, R., Leimer, U., Zweckbronner, I., Jakubek, C., Grünberg, J. and Haass, C. (1997) Human presenilin-1, but not familial Alzheimer's disease (FAD) mutants, facilitate *Caenorhabditis elegans* Notch signalling independently of proteolytic processing. *Genes Funct.*, **1**, 149–159.
- Benkovic, S.A., McGowan, E.M., Rothwell, N.J., Hutton, M., Morgan, D.G. and Gordon, M.N. (1997) Regional and cellular localization of presenilin-2 RNA in rat and human brain. *Exp. Neurol.*, **145**, 555–64.
- Blanchard, V., Czech, C., Bonici, B., Clavel, N., Gohin, M., Dalet, K., Revah, R., Pradier, L., Imperato, A. and Moussaoui, S. (1997) Immunohistochemical analysis of presenilin 2 expression in the mouse brain: distribution pattern and co-localization with presenilin 1 protein. *Brain Res.*, **758**, 209–17.
- Blaumueller, C.M., Qi, H., Zagouras, P. and Artavanis-Tsakonas, S. (1997) Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. *Cell*, **90**, 281–91.

- 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 1–42/1–40 Ratio *In Vitro* and *In Vivo*. *Neuron*, **17**, 1005–1013.
- Boulianne, G.L., Livne-Bar, I., Humphreys, J.M., Liang, Y, Lin, C., Rogaev, E. and St George-Hyslop, P. (1997) Cloning and characterization of the Drosophila presenilin homologue. *NeuroReport*, **8**, 1025–1029.
- Citron, M., Westaway, D., Xia, W, Carlson, G., Diehl, T., Levesque, G., Johnson-Wood, K., Lee, M., Seubert, P., Davis, A., Kholodenko, D., Motter, R., Sherrington, R., Perry, B., Yao, H., Strome, R., Lieberburg, I., Rommens, J., Kim, S., Scheme, D., Fraser, P., St George Hyslop, P., and Selkoe, D.J. (1997) Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid β -protein in both transfected cells and transgenic mice. *Nature Medicine*, **3**, 67–72.
- Daigle, I., and Li, C. (1993) *apl-1*, a *Caenorhabditis elegans* gene encoding a protein related to the human β -amyloid protein precursor. *Proc. Natl. Acad. Sci. USA*, **90**, 12045–12049.
- Doan, A., Thinakaran, G., Borchelt, D.R., Slunt, H.H., Ratovitsky, T., Podlisny, M., Selkoe, D.J., Seeger, M., Gandy, S.E., Price, D.L., and Sisodia, S.S. (1996) Protein Topology of Presenilin 1. *Neuron*, **17**, 1023–1030.
- Duff, K., Eckman, C., Zehr, C., Yu, X., Prada, C.M., Perez-tur, J., Hutton, M., Buee, L., Harigaya, Y, Yager, D., Morgan, D., Gordon, M.N., Holcomb, L., Refolo, L., Zenk, B., Hardy, J. and Younkin, S. (1996) Increased amyloid-beta 42 (43) in brains of mice expressing mutant presenilin 1. *Nature*, **383**, 710–713.
- Elder, G.A., Tezapsidis, N., Carter, J., Shioi, J., Bouras, C., Li, H.C., Johnston, J.M., Efthimiopoulos, S., Friedrich, V.L., Jr., and Robakis, N.K. (1996) Identification and neuron specific expression of the S182/presenilin I protein in human and rodent brains. *J. Neurosci. Res.*, **45**, 308–20.
- Favello, A., Hillier, L., and Wilson, R.K. (1995) Genomic DNA sequencing methods. *Methods Cell BioL*, **48**, 551–69.
- Ferguson, E.L., and Horvitz, H.R. (1985) Identification and Characterization of 22 Genes that Affect the Vulval Cell Lineages of the Nematode *Caenorhabditis elegans*. *Genetics*, **110**, 17–72.
- Greenwald, I., Sternberg, P.W. and Horvitz, H.R. (1983) The *lin-12* locus specifies cell fates in *Caenorhabditis elegans*. *Cell*, **34**, 435–444.
- Greenwald, I. (1989) Cell-cell interactions that specify certain cell fates in *C. elegans* development. *Trends Genetics*, **5**, 237–241.
- Greenwald, I., and Seydoux, G. (1990) Analysis of gain-of-function mutations of the *lin-12* gene of *Caenorhabditis elegans*. *Nature*, **346**, 197–199.
- Hardy, J. (1997a) The Alzheimer family of diseases: Many etiologies, one pathogenesis? *Proc. Natl Acad. Sci. USA*, **94**, 2095–2097.
- Hardy, J. (1997b) Amyloid, the presenilins and Alzheimer's disease. *Trends Neurosci.*, **20**, 154–159.
- Hong, C.S., and Koo, E.H. (1997) Isolation and characterization of a *Drosophila* presenilin homolog. *NeuroReport*, **8**, 665–668.
- Joutel, A., Corpechot, C., Ducros, A., Vahedi, K., Chabriat, H., Mouton, P., Alamowitch, S., Domenga, V., Cecillion, M., Marechal, E., Maciazek, J., Vayssiere, C., Cruaud, C., Cabanis, E.A., Ruchoux, M.M., Weissenbach, J., Bach, J.F., Bousser, M.G., and Tournier-Lasserre, E. (1996) NotchS mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *Nature*, **383**, 707–10.
- Kovacs, D.M., Fausett, H.J., Page, K.I., Kim, T.W., Moir, R.D., Merriam, D.E., Hollister, R.D., Hallmark, O.G., Mancini, R., Felsenstein, K.M., Hyman, B.T., Tanzi, R.E., and Wasco, W. (1996) Alzheimer-associated presenilins

- 1 and 2: Neuronal expression in brain and localization to intracellular membranes in mammalian cells. *Nature Medicine*, **2**, 224–229.
- Lemere, C.A., Lopera, F., Kosik, K.S., Lendon, C.L., Ossa, J., Saido, T.C., Yamaguchi, H., A, R., Martinez, A., Madrigal, L., Hincapie, L., L., J.C.A., Anthony, D.C., Koo, E.H., Goate, A.M., Selkoe, D.J. and Arango V, J.C. (1996) The E208A presenilin 1 Alzheimer mutation produces increased A β 42 deposition and severe cerebellar pathology. *Nature Medicine*, **2**, 1146.
- Levitan, D., Doyle, T.G., Brousseau, D., Lee, M.K., Thinakaran, G., Slunt, H.H., Sisodia, S.S., and Greenwald, I. (1996) Assessment of normal and mutant human presenilin function in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA*, **93**, 14940–14944.
- Levitan, D., and Greenwald, I. (1995) Facilitation of *lin-12*-mediated signalling by *sel-12*, a *Caenorhabditis elegans* S182 Alzheimer's disease gene. *Nature*, **377**, 351–354.
- Levy-Lahad, E., Wijsman, E.M., Nemens, E., Anderson, L., Goddard, K.A.B., Weber, J.L., Bird, T.D. and Schellenberg, G.D. (1995a) A Familial Alzheimer's Disease Locus on Chromosome 1. *Science*, **269**, 970–973.
- Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D.M., Oshima, J., Pettingell, W.H., Yu, C., Jondro, P.D., Schmidt, S.D., Wang, K., Crowley, A.C., Fu, Y.H., Guenette, S.Y., Galas, D., Nemens, E., Wijsman, E.M., Bird, T.D., Schellenberg, G.D., and Tanzi, R.E. (1995b) Candidate gene for the Chromosome 1 Familial Alzheimer's Disease Locus. *Science*, **269**, 973–977.
- L'Hernault, S.W. and Arduengo, P.M. (1992) Mutation of a Putative Sperm Membrane Protein in *Caenorhabditis elegans* Prevents Sperm Differentiation but Not Its Associated meiotic Divisions, *J. Cell Biol.*, **119**, 55–68
- Li, X., and Greenwald, I. (1996) Membrane Topology of the *C. elegans* SEL-12 Protein. *Neuron*, **17**, 1015–1021.
- Moussaoui, S., Czech, C., Pradier, L., Blanchard, V., Bonici, B., Gohin, M., Imperato, A., and Revah, F. (1996) Immunohistochemical analysis of presenilin-1 expression in the mouse brain. *FEBS Lett.*, **383**, 219–22.
- Pan, D., and Rubin, G.M. (1997) Kuzbanian controls proteolytic processing of Notch and mediates lateral inhibition during Drosophila and vertebrate neurogenesis. *Cell*, **90**, 271–80.
- Quarteronnet, D., Pradier, L., Czech, C., Delalonde, L., Burgevin, M.C., Doble, A., and Petitet, F. (1996) Localization of presenilin-1 mRNA in rat brain. *Neuroreport*, **7**, 2587–2591.
- Rogaev, E.I., Sherrington, R., Rogaeva, E.A., Levesque, G., Ikeda, M., Liang, Y., Chi, H., Lin, C., Holamn, K., Tsuda, T., Mar, L., Sorbi, S., Nacmias, B., Piacentini, S., Amaducci, L., Chumakkov, I., Cohen, D., Lannfelt, L., Fraser, P.E., Rommens, J.M. and St. George-Hyslop, P. (1995) Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. *Nature*, **376**, 775–778.
- Selkoe, D.J. (1996) Amyloid β -Protein and the Genetics of Alzheimer's Disease. *The Journal of Biological Chemistry*, **271**, 18295–18298.
- Seydoux, G., and Greenwald, I. (1989) Cell autonomy of *lin-12* function in a cell fate decision in *C. elegans*. *Cell*, **57**, 1237–1245.
- Shen, J., Bronson, R.T., Chen, D.R., Xia, W., Selkoe, D.J. and Tonegawa, S. (1997) Skeletal and CNS Defects in Presenilin-1-Deficient Mice. *Cell*, **89**, 629–639.
- Sherrington, R., Rogaev, E.I., Linag, Y., Rogaeva, E.A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., and St. George-Hyslop, P. (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature*, **375**, 754–760.
- Silva, H.A.R.D., and Patel, A.J. (1997) Presenilins and early-onset familial Alzheimer's disease. *NeuroReport*, **8**, 1–12.
- Takahashi, H., Murayama, M., Takashima, A., Mercken, M., Nakazato, Y., Noguchi, K., and Imahori, K. (1996) Molecular cloning and expression of the rat homologue of presenilin-1. *Neurosci Lett*, **206**, 113–6.

- Takano, T., Sahara, N., Yamanouchi, Y. and Mori H. (1997) Assignment of Alzheimer's presenilin-2 (PS-2) gene to 1q42.1 by fluorescence *in situ* hybridization *Neurosci. Lett.*, **221**, 205–207.
- Tanzi, R.E., Kovacs, D.M., Kim, T.W., Moir, R.D., Guenette, S.Y., and Wasco, W. (1996) The Gene Defects Responsible for Familial Alzheimer's Disease. *Neurobiology of Disease*, **3**, 159–168.
- Tsujimura, A., Yasojima, K., and Hashimoto-Gotoh, T. (1997) Cloning of Xenopus presenilin- α and - β cDNAs and Their Differential Expression in Oogenesis and Embryogenesis. *Biochemical And Biophysical Research Communications*, **231**, 392–396.
- Weidemann, A., Paliga, K., Dürrwang, U., Czech, C., Evin, G., Masters, C.L., and Beyreuther, K. (1997) Formation of stable complexes between two Alzheimer's disease gene products : Presenilin-2 and β -amyloid precursor protein. *Nature Medicine*, **3**, 328–332.
- Wilkinson, H.A., Fitzgerald, K., and Greenwald, I. (1994) Reciprocal Changes in Expression of the Receptor *lin-12* and Its Ligand *lag-2* Prior to Commitment in a *C. elegans* Cell Fate Decision. *Cell*, **79**, 1187–1198.
- Wilson, R., Ainscough, R., Anderson, K., Baynes, C., Berks, M., Bonfield, J., Burton, J., Connell, M., Copsey, T., Cooper, J., Coulson, A., Craxton, M., Dear, S., Du, Z., Durbin, R., Favello, A., Fraser, A., Fulton, L., Gardner, A., Green, P., Hawkins, Z., Hillier, L., Jier, M., Johnston, L., Hones, M., Kershaw, J., Kirsten, J., Laisster, N., Latreille, P., Lightning, J. *et al.* (1994) 2.2 Mb of contiguous nucleotide sequence from chromosome III of *C. elegans*. *Nature*, **368**, 32–38.
- Wong, P.C., Zheng, H., Chen, H., Becher, M.W., Sirinathsinghji, D.J.S., Trumbauer, M.E., Chen, H.Y., Price, D.L., Pleog, L.H.T.V.d., and Sisodia, S.S. (1997) Presenilin1 is required for Notch1 and Dll1 expression in the paraxial mesoderm. *Nature*, **387**, 288–292.
- Xia, W., Zhang, J., Perez, R., Koo, E.H., and Selkoe, D.J. (1997) Interaction between amyloid precursor protein and presenilins in mammalian cells: Implications for the pathogenesis of Alzheimer disease. *Proc. Natl Acad. Sci. USA*, **94**, 8208–8213.
- Zheng, H., Jiang, M., Trumbauer, M.E., Sirinathsinghji, D.J.S., Hopkins, R., Smith, D.W., Heavens, R.P., Dawson, G.R., Boyce, S., Conner, M.W., Stevens, K.A., Slunt, H.H., Sisodia, S.S., Chen, H.Y., and der Ploeg, L. (1995) β -Amyloid Precursor Protein-Deficient Mice Show Reactive Gliosis and Decreased Locomotor Activity. *Cell*, **81**, 525–531.
- Zhou, J., Liyanage, U., Medina, M., Ho, C., Simmons, A.D., Lovett, M., and Kosik, K.S. (1997) Presenilin 1 interaction in the brain with a novel member of the Armadillo family. *NeuroReport*, **8**, 2085–2089.

13.

THE PROCESSING OF THE AMYLOID-PRECURSOR- PROTEIN (APP) IN PRESENILIN-1 DEFICIENT NEURONS

PAUL SAFTIG¹, DIETER HARTMANN², WIM ANNAERT³, KATHLEEN
CRAESSAERTS³, FRED VAN LEUVEN³ and BART DE STROOPER³

¹*Zentrum Biochemie und Molekulare Zellbiologie, Abteilung Biochemie II, Universität
Göttingen, Germany*

²*Anatomisches Institut, Universität Kiel, Germany*

³*Flemish Institute for Biotechnology (VIB4) and the Experimental Genetics Group,
Center for Human Genetics, K.U.Leuven, Belgium*

A distinguishing feature of Alzheimer's disease (AD) is the deposition of amyloid plaques in the brain, which arise by abnormal accumulation of β A4 peptide (Selkoe, 1991). The 39–43-residue β A4-Amyloid peptide, the main component of the amyloid plaque in the brain of Alzheimer's disease patients is generated from amyloid precursor protein (APP) by proteolytical processing (Haass and Selkoe, 1993).

The cause of the sporadic and most frequent form of the disease is still unknown. Familial, early onset Alzheimer's Disease is either caused by point mutations in the amyloid precursor protein gene on chromosome 21 (Goate *et al.*, 1991), in the presenilin 2 (PS2) gene on chromosome 1 (Rogaev *et al.*, 1995; Levy-Lahad *et al.*, 1995), or, most frequently, in the presenilin 1 (PS1) gene on chromosome 14 (Sherrington *et al.*, 1995; Alzheimer's Disease Collaborative Group, 1995; Van Broeckhoven, 1995). Point mutations in the presenilins are responsible for most of the familial forms of the disease (Van Broeckhoven, 1995). Given the similar neuropathology of the familial and the sporadic forms of AD, understanding the biochemical and metabolic relationships between the presenilins and APP will also lead to insight into the pathogenesis of sporadic AD.

Since the identification of PS1, considerable progress has been made in defining its structure and intracellular localisation. Immunocytochemical studies revealed that PS1 is localized mainly in the endoplasmic reticulum and, to a lesser extent, in the Golgi compartment (Kovacs *et al.*, 1996; Walter *et al.*, 1996; De Strooper *et al.*, 1997). PS1 is a transmembrane spanning protein containing between seven and nine candidate transmembrane domains and a hydrophilic loop region (Sherrington *et al.*, 1995). The N-terminal domain, loop, and C-terminal domains of PS1 are orientated towards the cytoplasm (Doan *et al.*, 1996; De Strooper *et al.*, 1997; Lehmann *et al.*, 1997; Li and Greenwald, 1996). PS1 is proteolytically cleaved to generate two fragments of 17 and 27 kDa (Thinakaran *et al.*, 1996; Podlinski *et al.*, 1997). PS 1 is expressed in a variety of tissues, including the embryonic and adult brain (Sherrington *et al.*, 1995; Lee *et al.*, 1996; Berezovka *et al.*, 1997). In brain it is primarily expressed in neurons, with highest expression in the cerebellum and hippocampus (Kovacs *et al.*, 1996; Lee *et al.*, 1996; Suzuki *et al.*, 1996).

While convincing evidence exists that PS1 mutations cause increased production of amyloidogenic βA_{41-42} peptide (Scheuner *et al.*, 1996; Duff *et al.*, 1996; Borchelt *et al.*, 1996; Citron *et al.*, 1997), it remains unclear whether this reflects a gain or a loss of function (Levitan *et al.*, 1996; Baumeister *et al.*, 1997). To tackle this problem directly, we generated PS 1 deficient mice and analysed the metabolism of APP in mixed brain cultures derived from E14 embryos (De Strooper *et al.*, 1998).

TARGETING OF THE PRESENILIN-1 GENE IN EMBRYONIC STEM (ES) CELLS AND GENERATION OF PS1 HOMOZYGOUS MUTANT MICE

A genomic lambda-fixII library of mouse SV-129 DNA (Stratagene, LaJolla, CA) was screened with a 0.8 kb murine PS1 cDNA fragment spanning exon 7 to 11. Six overlapping genomic clones were isolated, subcloned, characterized and the exons and exon-intron boundaries were sequenced. The PS1 mouse gene was mapped using the interspecific backcross DNA panel (Jackson laboratory; Rowe *et al.*, 1994) to the distal mouse chromosome 12 (38.0 cM) within a region of syntheny with human chromosome 14q24.3. To generate a null mutation in PS1 a targeting vector with 5.6 kb homology to the PS1 gene locus was constructed. A unique Sall site at the cDNA position 576 of PS1 (the ATG is taken as codon 1) was introduced by site directed mutagenesis and the neomycin phosphotransferase gene (Neo) under the control of the PGK promoter (Thomas and Capecchi, 1987) was inserted. The Neo insertion leads to an interruption of the open reading frame (ORF) in exon 7 corresponding to a position that is 10 amino acid residues carboxyterminal of the third transmembrane domain of PS1.

The linearized targeting construct (Figure 1a) was introduced into E-14-1 ES cells (Hooper *et al.*, 1987) and G418-resistant colonies were analysed by Southern blotting. In nine out of 78 independent clones tested, an additional KpnI (6.8 kb in wildtype and 4.5 kb in the mutant allele) and NcoI fragment (8.5 kb in wildtype and 7.6 kb in the mutant allele) was detected with the 5' external probe, indicating a homologous recombination event in one of the PS1 alleles (Figure 1b). The targeted ES-cell clones were microinjected into C57BL/6J blastocysts and a total of ten chimaeric males were generated. Of these ten chimaeras, nine transmitted the mutant allele to their offspring.

Offspring from heterozygous intercrosses were genotyped either by Southern Blotting (Figure 1c) or by PCR (Figure 1d). Heterozygous offspring were observed for more than 28 weeks. They did not show differences in phenotype or fertility as compared with wild-type littermates. Genotyping of 105

embryos after heterozygote crosses taken between day 14 and 16 revealed a frequency of 27% for homozygous mutants. No mutants were found in three litters after natural delivery. In complete agreement with the two independently generated PS-1 knockout models (Wong *et al.*, 1997; Shen *et al.*, 1997) we confirm that PS1 $-/-$ mice die late in embryogenesis.

FUNCTIONAL INACTIVATION OF THE PRESENILIN-1 GENE

To test for expression of presenilin-1 at mRNA and protein level, Northern blot analysis of whole embryo RNA and Western blotting of membrane fractions were performed. A 3 kb PS-1-specific mRNA was detectable in RNA from wild-type embryos, whereas no PS-1 transcript was detectable in RNA from homozygous mutant animals (Figure 1f). The level of PS1 mRNA is reduced in heterozygotes compared to wild-type embryos (Figure 1f). Western blot analysis using an affinity purified polyclonal antibody B17/2 against the loop domain of PS1 (De Strooper *et al.*, 1997) confirmed the absence of the 17 kD C-terminal fragment in homozygous mutants and a reduction in intensity in heterozygotes (Figure 1h). Absence of immunoreactivity for PS1 in presenilin-1 $-/-$ embryos was also demonstrated using an antibody against the N-terminus of PS1 (data not shown).

Northern and Western blot analyses and the embryonic lethality of the PS-1 mutant mice therefore all confirm that the PS-1 gene has been inactivated and that the homozygous mutant mice are devoid of PS-1.

PHENOTYPE OF PRESENILIN-1 MUTANT MICE AND CULTURED NEURONS

PS-1 mutant embryos display a severe growth retardation and skeletal malformation, which is most pronounced in the caudal regions and exemplified by a stubby tail (Figure 1e). Intraventricular and intraparenchymal bleeding was observed in most of the PS1 $-/-$ embryos with varying degrees of severity. Preliminary immunohistological analyses of 15 day old PS-1-deficient embryo brains indicated a marked reduction in the density of vascularization, most obvious in the regions of the hemispherical anlage ventral to the rhinal fissure and within the ganglionic eminence. The cortical plate of E15 appears more densely packed, while the delineation of its internal laminar and its radial organization are disturbed (Hartmann, unpublished data). The ventricular proliferative zones of both the lateral and the third ventricles at E15 exhibit signs of collapse, including the budding and shedding of groups of stem cells into the ventricular lumen (Hartmann, unpublished data), as has been described e.g. consecutive to sublethal irradiation (Bayer and Altman, 1991). To circumvent embryonic lethality of PS1 $-/-$ mice and to allow the biochemical analysis of APP processing, mixed brain cultures from embryos at day 14pc were prepared according to protocols previously used for hippocampal neurons (De Strooper *et al.*, 1995; Tienari *et al.*, 1996; Simons *et al.*, 1996). Morphologically the brain cultures derived from littermate embryos with the different genotypes were indistinguishable (Figure 1g; shown for PS1 $+/-$ and PS1 $-/-$ cultures). Cell yields were comparable between genotypes with a slight reduction in PS1 $-/-$ embryos. The cultures derived from littermate embryos with the different genotypes contained almost only, if not exclusively, neuronal cells as evaluated by phase contrast and fluorescence microscopy using antibodies against Tau, Map2 and GFAP (Figure 2; shown for Tau).

METABOLISM OF ENDOGENOUSLY EXPRESSED MOUSE APP IN
PRESENILIN-1 DEFICIENT NEURONS

PS1-deficient and control cultures were metabolically labelled (Saftig *et al.*, 1996) and amyloid peptide and carboxyterminal fragments from endogenously expressed mouse APP were

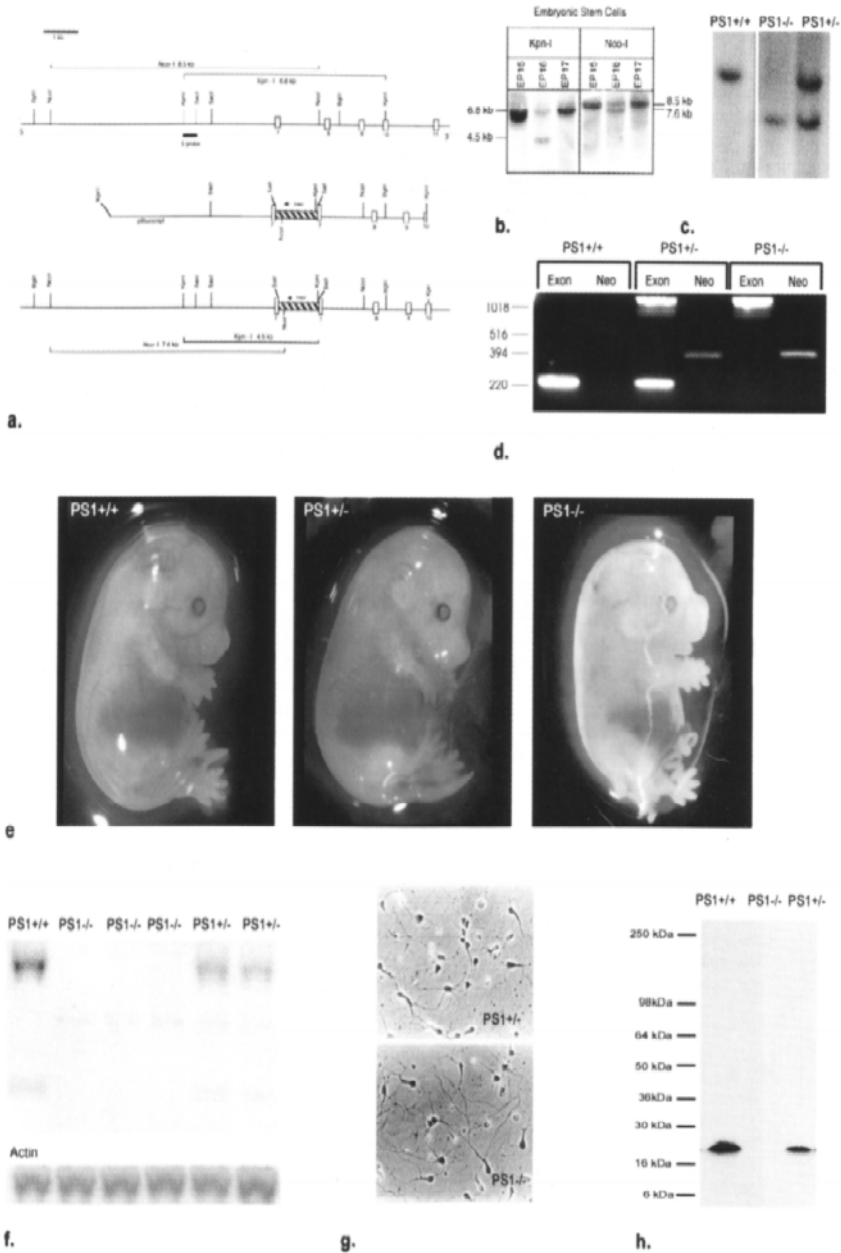


Figure 1. Targeted inactivation of the presenilin-1 mouse gene.

(A) Strategy for the inactivation of the PS-1 gene by homologous recombination in embryonic stem cells. Partial genomic structure and restriction map of the PS1 gene (upper). Exons are numbered and indicated by open boxes. Introns, 5' and 3' flanking regions are indicated by a solid line. The black bar designates the 5' probe used for Southern blot analyses. Targeting vector (middle) with 5.6 kb homology to the PS1 gene locus. A unique *SalI* site was introduced by site directed mutagenesis in exon 7 and the neomycin phosphotransferase gene (Neo) under the control of the PGK promoter was inserted. Predicted PS-1 gene locus (lower) after homologous recombination. The 5' *KpnI*—*SacI* external probe detects a 6.8 and 4.5 kb *KpnI* and 8.5 and 7.6 kb *NcoI* restriction fragment in mutated (lower) and wild-type (upper) alleles, respectively.

(B) Southern Blot analysis of ES cell clones. The 5' probe was hybridized to *KpnI* or *NcoI*-digested DNA from three ES cell clones (EP-15, EP-16, EP-17) with a targeted allele as indicated by an additional 4.5 kb *KpnI* and 7.6 kb *NcoI* fragment, respectively.

(C) Southern blot analysis of mouse embryos. A 6.8 kb *KpnI* fragment in wildtype (PS1+/+) and heterozygote (PS1+/-) and a 4.5 kb fragment in homozygote (PS1-/-) and heterozygote (PS1 +/-) is visualized with the 5' *KpnI*-*SacI* probe.

(D) PCR analysis of mouse embryos. The primers used to detect the neomycin cassette have been described (Saftig *et al.*, 1996). PS-1-exon7 specific PCR (primers PS1-14: 5'-gggaagtatttaagacctacaatggt-3' and PS1-15: 5'-catatactgaaatcacagccaag-3') amplifies a 222bp fragment in the wild type allele and a 1.4 kb fragment in the targeted allele due to insertion of the neomycin cassette.

(E) 14 day old littermate embryos used for generation of neuronal cultures. Lateral view of wild type, heterozygote and null embryos showing the growth retardation and skeletal malformation.

(F) The PS1-gene is inactivated in homozygous mutant embryos. Northern blot analysis of PS1 mRNA expression. Total embryo RNA (10 μ g) was hybridized to a PS1 cDNA probe and subsequently a murine actin cDNA as internal control. PS1 mRNA (3.0 kb) was absent in three embryos examined.

(G) Neuronal cultures of PS1 +/- and PS1 -/- after 4 days *in vitro*. The cultures were generated from brain of E-14 embryos (De Strooper *et al.*, 1995; Tienari *et al.*, 1996; Simons *et al.*, 1996; Saftig *et al.* 1996).

(H) PS1 protein expression in a Western blot. Membrane fractions (30 μ g/lane) from PS1 +/+, PS1 +/- and PS1 -/- embryos were analysed with a purified polyclonal antibody B17/2 against the loop domain of PS1 (De Strooper *et al.*, 1997). In PS1-mutants (-/-) no PS1 protein was detectable. (See Colour Plate III)

immunoprecipitated and analysed in SDS-PAGE. Apart from a strong inhibition of β amyloid peptide and the p3-fragment secretion in the culture medium an accumulation of carboxyterminal fragments of APP in the PS1-/-cultures was observed (De Strooper *et al.*, 1998). An increasing amount of carboxyterminal APP fragments in presenilin-1 deficient mice could also been demonstrated in a Western blot experiment using entire mouse brains (Figure 3c), respectively. These findings already indicated a direct role of PS1 in the amyloidogenic processing of APP. To further quantitatively analyse this effect human wild type APP, and human APP containing the London (Val 642 to Ile) or Swedish (Lys-595 to Asn and Met-596 to Leu) type of clinical mutations were expressed in the neurons using Semliki Forest Virus (De Strooper *et al.*, 1998).

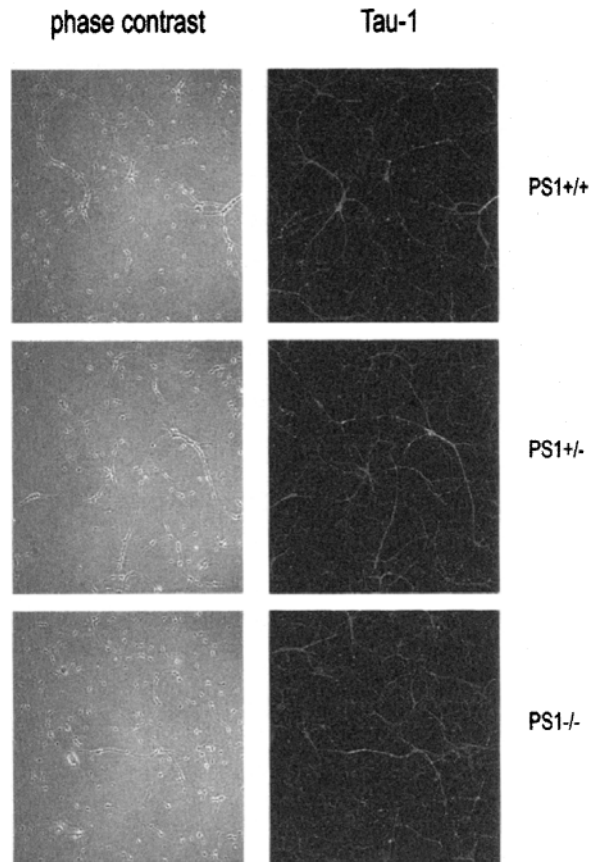


Figure 2. Brain cultures from PS1 $+/+$, PS1 $+/-$ and PS1 $-/-$ embryos at day 14pc are morphologically not distinguishable and contain almost exclusively neuronal cells. Neuronal cells were obtained according to protocols previously established for hippocampal neurons (De Strooper *et al.*, 1995; Tienari *et al.*, 1997; Simons *et al.*, 1996; Saftig *et al.*, 1996). Proliferation of non-neuronal cells was prevented by adding cytosine arabinoside. Cells were fixed with 4% paraformaldehyde, permeabilized with ethanol and stained with Tau-1 mAb and FITC conjugated secondary antibodies. The livers of the embryos were used for the genotyping of the culture.

METABOLISM OF VIRALLY EXPRESSED HUMAN APP IN PRESENILIN-1 DEFICIENT NEURONS

Using a panel of well-characterized APP antibodies, all known aspects of the proteolytic processing of APP in PS1 $-/-$ cells were analysed. No differences between genotypes were found concerning the secretion of APP ectodomain (APPs), indicating normal α - and/or β -secretase processing of APP (De Strooper *et al.*, 1998). As already observed for endogenous APP, β -amyloid peptide (Figure 3b) and p3 secretion (not shown) were significantly reduced in PS1 $-/-$ cells (De Strooper *et al.*, 1998). The fivefold decrease in amyloid peptide secretion was accompanied by a concomittant twofold increase in β -secretase and a fivefold increase in α -secretase cleaved carboxyterminal fragments in the cell

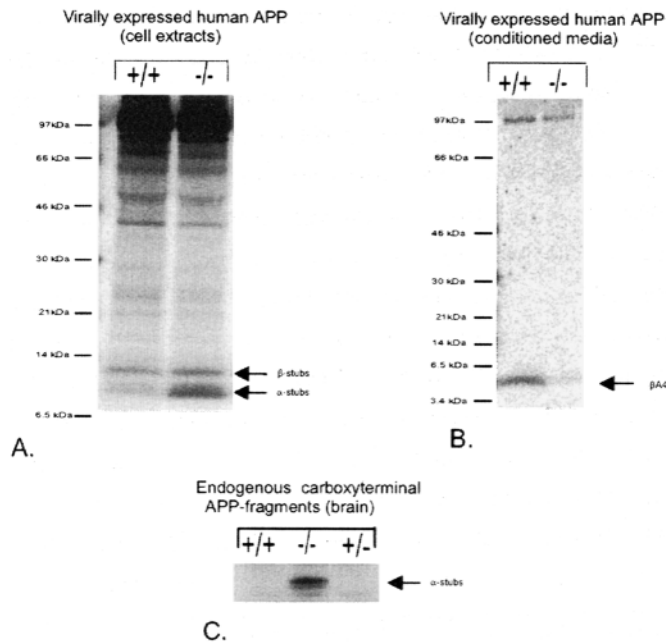


Figure 3. Processing of Amyloid Precursor Protein in PS1-deficient neurons.

(A) Neuronal cultures were infected with recombinant Semliki Forest Virus driven expression of human wild type APP and were labeled with ^{35}S -methionine for 4 hours. APP and APP carboxyterminal fragments were immunoprecipitated from the cell extracts using anti serum APP675–695 (De Strooper *et al.*, 1998). Note the increased amount of α - and β -secretase cleaved fragments in the PS1 deficient neurons.

(B) Secreted amyloid peptide was immunoprecipitated with anti-APP 597–612 from the conditioned media. This antibody does not immune precipitate the p3-fragment (De Strooper *et al.*, 1995). βA4 secretion is strongly inhibited in PS1 $-/-$ neurons.

(C) Western blot of membrane fractions (50 μg /lane) from PS1 $+/+$, PS1 $+/-$ and PS1 $-/-$ embryonic brains after immunoprecipitation with the carboxyterminal specific APP antibody 675–695 (De Strooper *et al.*, 1998) using the same antiserum. Note the accumulation of carboxyterminal fragments in PS1 $-/-$ embryonic brain.

extracts (Figure 3a; De Strooper *et al.*, 1998). The same experiments using the clinical APP mutants confirmed the findings with the wild type APP (De Strooper *et al.*, 1998). Pulse chase experiments confirmed that PS1 deficiency specifically decreased the turnover of the membrane associated fragments of APP. In PS1 $-/-$ neurons newly generated carboxyterminal fragments of APP accumulated and remained stable often up to four hours of chase in PS1 $-/-$ cells, whereas in PS1 $+/+$ neurons these fragments are rapidly turned over (De Strooper *et al.*, 1998).

The production of both $\beta\text{A41-40}$ and $\beta\text{A41-42}$ was determined by means of specific Elisa's and was shown to be decreased 3.6 fold and 3.2 fold, respectively (De Strooper *et al.*, 1998). This demonstrates that the two putative γ -secretases (Citron *et al.*, 1996; Klafki *et al.*, 1996; Hartmann *et al.*, 1997) are equally affected by the PS-1 null mutation.

PS 1 IS A FACILITATOR OF THE CLEAVAGE OF THE INTEGRAL MEMBRANE DOMAIN OF APP WITH ANALOGY TO THE SREBP-SCAP INTERACTIONS

The described altered metabolism of APP in PS-1 deficient neurons conclusively demonstrates that PS1 is involved in the normal proteolytic cleavage of the carboxyterminal fragments of APP. PS1 appears to be directly involved in the γ -secretase cleavage step of APP after the α - and β -secretase specific fragments have been generated (Figure 4b). Since no structural or sequence homology between PS1 and any protease domain known so far has been described it appears unlikely that PS1 is itself (one of) the γ -secretase(s). Moreover, the fact that even in PS1 $-/-$ cells residual amyloid peptide secretion persists, suggests that PS1 is indirectly involved in γ -secretase cleavage, i.e. as a cofactor in this processing. The question whether PS2 is responsible for this residual amyloid peptide secretion will be answered with the help of PS2 and PS1/PS2 double knockout animals. The demonstration that APP and presenilins might interact directly with each other (Weidemann *et al.*, 1997; Xia *et al.*, 1997) and the fact that they are at least temporally colocalized in the endoplasmic reticulum and Golgi apparatus (Kovacz *et al.*, 1996; Walter *et al.*, 1996; De Strooper *et al.*, 1997) strongly supports our model that PS1 acts as a facilitator of the proteolytic activity enabling cleavage of the integral membrane domain of APP. In a recent review Brown and Goldstein discussed some analogies between APP processing and the processing of the membrane bound transcription factors sterol regulatory element binding proteins (SREBPs). The authors suggest that the SREBP site 1 protease is analogous to the APP β -secretase and that the SREBP site 2 cleavage, which also cleaves in the intramembrane domain, resembles the subsequent γ -secretase cleavage of APP (Figure 4a, b). The SREBP cleavage activating protein (SCAP) and the presenilins are similar in their polytopic membrane character, cellular localisation, stimulatory action, and activation by point mutations (Brown and Goldstein, 1997). SCAP and PS1 could belong to a novel class of protein-chaperones that are responsible for the access to or exposure of protein domains for proteolytic processing. The fact that the activity of SCAP is regulated by intracellular cholesterol levels (Hua *et al.*, 1997), raises intriguing questions with regard to the regulation of PS1 and to the possible links to the indirect effect of Apo E4 on the pathology in AD, via the possible modulation of γ -secretase or β -secretase processing of APP (Corder *et al.*, 1993).

Beside the analogies between β APP and SREBPs processing also two differences should be noted. First, the membrane orientation is reversed: While the NH_2 -terminus of SREBPs is cytoplasmic, it is the COOH -terminus of APP that faces the cytoplasm (Figure 4). Furthermore SCAP is acting also on the proteolytic cleavage of site 1 (the homologue of the β -secretase site in APP), whereas PS1 did only show an effect on the cleavage site of the intramembrane domain ("site 2") of APP. The idea that PS1 is involved in the transport of membrane anchored carboxyterminal APP stubs towards the γ -secretase (s) containing subcellular compartment could alternatively explain the requirement of PS1 for the intramembraneous cleavage of APP.

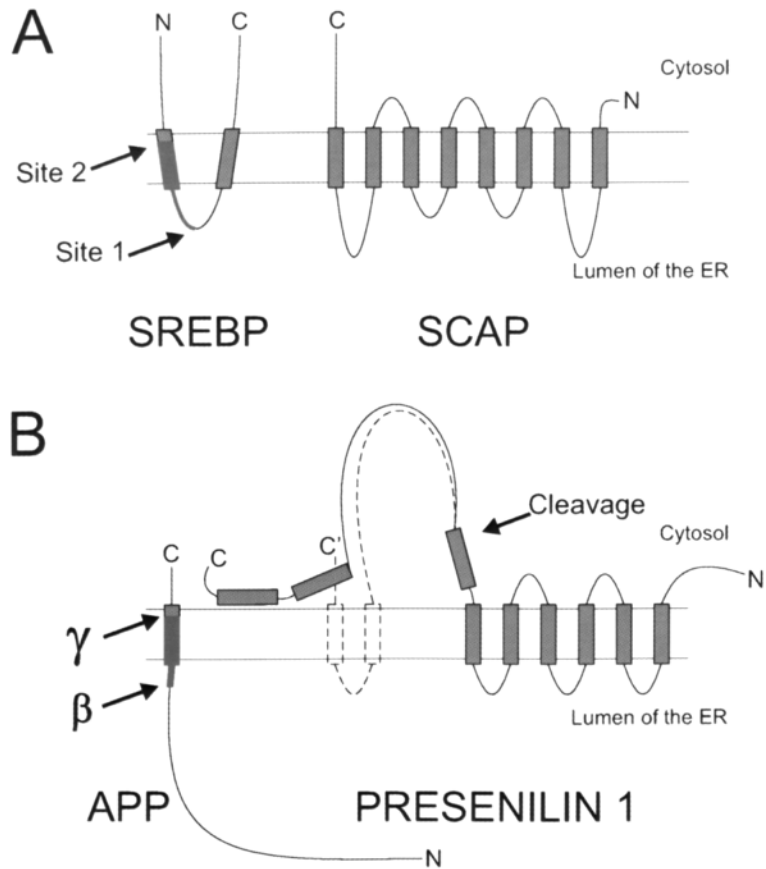


Figure 4. APP/Presenilin and SREBP/SCAP analogies. A hypothetical representation of SCAP and Presenilin as multiple spanning proteins residing in the endoplasmic reticulum. SCAP is a facilitator of SREBP processing (A), while presenilin is a facilitator of APP processing (B). For further details: see text. (See Colour Plate IV)

FUTURE PROSPECTS: IDENTIFICATION OF THE γ -SECRETASE(S)

Having demonstrated that PS1 is a putative cofactor in the γ -secretase(s) mediated intramembrane cleavage of APP it is obvious that the next step should be the identification of the actual protease(s) catalysing the cleavage of the APP-transmembrane substrate. Coimmunoprecipitation studies with PS1 and the screening of interacting proteins using the yeast 2-hybrid system are two possible strategies to identify possible γ -secretase candidates.

In this respect the recent identification of the Site-2 protease that mediates the intramembrane cleavage of the SREBPs using complementation cloning of the cDNA that corrects the defective cleavage in M19 cells is very promising. A hydropathy plot suggested that the cloned cDNA contains 4-6 transmembrane regions and a classical zinc metalloproteinase motif (Goldstein, personal

communication). It will be very interesting to determine whether the identified Site-2 protease also affects the processing of APP.

Inhibition of the γ -secretase itself and of PS1 could decrease the amyloid peptide production in neurons and could possibly provide a target for anti-amyloidogenic therapy in sporadic Alzheimer's Disease. However, the consequences of reducing the activity of γ -secretase and/or PS1 for the adult brain, remain to be evaluated e.g. in conditionally targeted PS1 mice.

REFERENCES

- Alzheimer, S.D.C.G. (1996) The structure of the presenilin 1 (S182) gene and identification of six novel mutations in early onset AD families. *Nature Genet.*, **11**, 219–222.
- Baumeister, R., Leimer, U., Zweckbronner, I., Jakubek, C., Gruenberg, J., and Haas, C. (1997) The sel-12 phenotype of *C. elegans* is rescued independent of proteolytic processing by wt but not mutant Presenilin. *Genes and Function*, **1**, 149–159.
- Bayer, S., and Altman, J. (1991) *Neocortical development*. Raven Press, New York.
- Berezovka, O., Xia, M., Page, K., Wasco, W., Tanzi, R., and Hyman, N. (1997) Developmental regulation of presenilin mRNA expression parallels Notch expression. *J. Neuropath. Exp. Neurol.*, **56**, 40–44.
- 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 1–42/1–40 ratio *in vitro* and *in vivo*. *Neuron*, **17**, 1005–1013.
- Brown, M.S., and Goldstein J.L. (1997) The SREBP Pathway: Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell*, **89**, 331–340.
- Citron, M., Diehl, T.S., Gordon, G., Biere, A.L., Seubert, P., and Selkoe, D.J. (1996) Evidence that the 42- and 40-amino acid forms of amyloid beta protein are generated from the beta-amyloid precursor protein by different protease activities. *Proc. Natl Acad. Sci. USA*, **93**, 13170–13175.
- Citron, M., Westaway, D., Xia, W., Carlson, G., Diehl, T., Levesque, G., Johnson-Wood, K., Lee, M., Seubert, P., Davis, A., Kholodenko, D., Motter, R., Sherrington, R., Perry, B., Yao, H., Strome, R., Lieberburg, I., Rommens, J., Kim, S., Schenk, D., Fraser, P., St George-Hyslop, P., and Selkoe, D.J. (1997) Mutant presenilins of Alzheimer's disease increase production of 42 residue amyloid β -protein in both transfected cells and transgenic mice. *Nature Med.*, **3**, 67–72.
- 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 dosage of apolipoprotein E type 4 allele and the risk of Alzheimer's Disease in late onset families. *Science*, **261**, 921–923.
- De Strooper, B., Simons, M., Multhaup, G., van Leuven, F., Beyreuther, K., and Dotti, C.G. (1995) Production of intracellular amyloid-containing fragments in hippocampal neurons expressing human amyloid precursor protein and protection against amyloidogenesis by subtle amino acid substitutions in the rodent sequence. *EMBO J.*, **14**, 4932–4938.
- De Strooper, B., Beullens, M., Contreras, B., Levesque, L., Craessaerts, K., Cordell, B., Moechars, D., Bollen, M., Fraser, P., St. George Hyslop, P., and Leuven, F. Van (1997) Phosphorylation, subcellular localisation, and membrane orientation of the Alzheimer's disease-associated presenilins. *J. Biol Chem.*, **272**, 3590–3598.

- De Strooper, B., Saftig, P., Craessaerts, K., Vanderstichele H., Guhde, G., Annaert, W., von Figura, K., and van Leuven, F. (1998) Deficiency of Presenilin 1 inhibits the normal cleavage of Amyloid Precursor Protein *Nature*, in press.
- Doan, A., Thinakaran, G., Borchelt, D., Slunt, H.H., Ratovitsky, T., Podlisny, M., Selkoe, D.J., Seeger, M., Gandy, S.E., Price, D.L., and Sisodia, S.S. (1996) Protein topology of presenilin 1. *Neuron*, **17**, 1023–1030.
- Duff, K., Eckman, C., Zher, C., Yu, X., Prada, C.-M., Perez-Tur, J., Hutton, M., Buee, L., Hargaya, Y., Yager, D., Morgan, D., Gordon, M.N., Holcomb, L., Refolo, L., Zenk, B., Hardy, J., and Younkin, S. (1996) Increased amyloid- β 42(43) in brains of mice expressing mutant presenilin 1. *Nature*, **383**, 710–713.
- Goate, A., Chartier-Harlin, M.C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., Mant, R., Newton, P., Rooke, K., Roques, P., Talbot, C., Pericak-Vance, M., Roses, A., Williamson, Rossor, M., Owen, M., and Hardy, J. (1991) Segregation of a missense mutation in the amyloid precursor protein in familial Alzheimer's disease increases β -protein production. *Nature*, **349**, 704–706.
- Haas, C., and Selkoe, D.J. (1993) Cellular processing of β -amyloid precursor protein and the genesis of amyloid β -peptide. *Cell*, **75**, 1039–1042.
- 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 β 40/42 amyloid peptides. *Nat Med.*, **3**, 1016–1020.
- Hooper, M., Hardy, K., Handyside, A., Hunter, S., and Monk, M. (1987) HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. *Nature*, **326**, 292–295.
- Hua, X., Nohturfft, A., Goldstein, J.L., and Brown, M.S. (1997) Sterol resistance in CHO cells traced to point mutation in SREBP cleavage-activating protein. *Cell*, **87**, 415–426.
- Klafki, H., Abramowski, D., Swoboda, R., Paganetti, P.A., and Staufenbiel, M. (1996) The carboxyl termini of β -amyloid peptides 1–40 and 1–42 are generated by distinct gamma-secretase activities. *J. Biol. Chem.*, **271**, 28655–28659.
- Kovacs, D.M., Fausett, H.J., Page, K.J., Kim, T.W., Moir, R.D., Merriam, D.E., Hollister, R.D., Hallmark, O.G., Mancini, R., Felsenstein, K.M., Hyman, B.T., Tanzi, R.E., and Wasco, W. (1996) Alzheimer-associated presenilins 1 and 2: neuronal expression in brain and localization to intracellular membranes in mammalian cells. *Nature Med.*, **2**, 224–229.
- Lee, M.K., Slunt, H.H., Martin, L.J., Thinakaran, G., Kim, Gandy, S.E., Seeger, M., Koo, E., Price, D.L., and Sisodia, S.S. (1996) Expression of presenilin 1 and 2 (PS1 and PS2) in human and murine tissues. *J. Neurosci.*, **16**, 7513–7525.
- Lehmann, S., Chiesa, R., and Harris, D.A. (1997) Evidence for a six-transmembrane domain structure of presenilin 1. *J. Biol. Chem.*, **272**, 12047–12051.
- Levitan, D., Doyle, T.G., Brousseau, D., Lee, M.K., Thinakaran, G., Thinakaran, G., Slunt, H.H., Sisodia, S.S., and Greenwald, I. (1996) Assessment of normal and mutant human presenilin function in *Caenorhabditis elegans*. *Proc. Natl Acad. Sci. USA*, **93**, 14940–14914.
- Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D., Oshima, J., Pettingell, W.H., Yu, C.E., Jondro, P.D., Schmidt, S.D., Wang, K., Crowley, Y.-H., Guenette, S.Y., Galas, D., Nemens, E., Wijsman, E.M., Bird, T.D., Schellenberg, G.D., and Tanzi, R.E. (1995). Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science*, **269**, 973–977.
- Li, X., and Greenwald, I. (1996) Membrane topology of the *C. elegans* SEL-12 presenilin. *Neuron*, **17**, 1015–1021.
- Podlisny, M.B., Citron, M., Amarante, P., Sherrington, R., Xia, W., Zhang, J., Diehl, T., Levesque, G., Fraser, P., Haass, C., Koo, E.H., Seubert, P., St.-George-Hyslop, P., Teplow, D.B., and Selkoe, D.J. (1997) Presenilin proteins

- undergo heterogeneous endoproteolysis between Thr291 and Ala299 and occur as stable N- and C-terminal fragments in normal and Alzheimer brain tissue. *Neurobiol Dis.*, **3**, 325–337.
- Rogaev, E.I., Sherrington, R., Rogaeva, E.A., Levesque, G., Ikeda, M., Liang, Y., Chi, H., Lin, C., Holman, K., Tsuda, T., Mar, L., Sorbi, S., Nacmias, B., Placentini, S., Amuducci, L., Chumakov, I., Cohen, D., Lannfelt, L., Fraser, P.E., Rommens, J.M., and St George-Hyslop, P.H. (1995) Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. *Nature*, **376**, 775–778.
- Rowe, L.B., Nadeau, J.H., Turner, R., Frankel, W.N., Letts, V.A., Eppig, J.T., Ko, M.S., Thurston, S.J., and Birkenmeier, E.M. (1994) Maps from two interspecific backcross DNA panels available as community genetic mapping resource. *Mamm. Genome*, **5**, 253–274.
- Saftig, P., Peters, C., von-Figura, K., Craessaerts, K., Van-Leuven, F., and De-Strooper, B. (1996) Amyloidogenic processing of human amyloid precursor protein in hippocampal neurons devoid of cathepsin D. *J. Biol. Chem.*, **271**, 27241–27244.
- Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T.D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-Lahad, E., Viitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wasco, W., Lannfelt, L., Selkoe, D., and Younkin-S (1996) Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased *in vivo* by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nature Med.*, **2**, 864–870.
- Selkoe, D.J. (1991) The molecular pathology of Alzheimer's disease. *Neuron*, **6**, 487–498.
- Shen, J., Bronson, R.T., Chen, D.F., Xia, W., Selkoe, D., and Tonegawa, S. (1997) Skeletal and CNS defects in presenilin-1 deficient mice. *Cell*, **89**, 629–639.
- Sherrington, R., Rogaev, E. I., Liang, Y., Rogaeva, E., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., Tsuda, T., Mar, L., Foncin, J.F., Bruni, A.C., Montesi, M.P., Sorbi, S., Rainero, I., Pinessi, L.N., Chumakov, L., Pollen, D., Brookes, A., Sanseau, P., Polinsky, R.J., Wasco, W., DaSilva, H.A.R., Haines, J.L., Pericak-Vance, M.A., Tanzi, R.E., Roses, A.D., Fraser, P.E., Rommens, J.M., and St George-Hyslop, P.H. (1995) Cloning of a gene bearing missense mutations in early onset familial Alzheimer's Disease. *Nature*, **375**, 754–760.
- Simons, M., De Strooper, B., Multhaup, G., Tienari, P.J., Dotti, C.G., and Beyreuther, K. (1996) Amyloidogenic processing of the human amyloid precursor protein in primary cultures of rat hippocampal neurons. *J. Neurosci.*, **16**, 899–908.
- Suzuki, T., Nishiyama, K., Murayama, S., Yamamoto, A., Sato, S., Kanazawa, I., and Sakaki, Y. (1996) Regional and cellular presenilin 1 gene expression in human and rat tissues. *Biochem. Biophys. Res. Commun.*, **219**, 708–713.
- Thinakaran, G., Borchelt, D.R., Lee, M.K., Slunt, H.H., Spitzer, L., Kim, G., Ratovitsky, T., Davenport, F., Nordstedt, C., Seeger, M., Hardy, J., Levey, A.I., Gandy, S.E., Jenkins, N.A., Copeland, N.G., Price, D.L., and Sisodia, S.S. (1996) Endoproteolysis of presenilin 1 and accumulation of processed derivatives *in vivo*. *Neuron*, **17**, 181–190.
- Thomas, K.R., and Capecchi, M.R. (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell*, **51**, 503–512.
- Tienari, P.J., De Strooper, B., Ikonen, E., Simons, M., Weidemann, A., Czech, C., Hartmann, T., Ida, N., Multhaup, G., Masters, C.L., Van Leuven, F., Beyreuther, K., and Dotti, C.G. (1996) The β -amyloid domain is essential for axonal sorting of amyloid precursor protein. *EMBO J.*, **15**, 5218–5229.
- Van Broeckhoven, C. (1995) Presenilins and Alzheimer disease [news]. *Nat Genet.*, **11**, 230–232.
- Walter, J., Capell, A., Grunberg, J., Pesold, B., Schindzielorz, A., Prior, R., Podlisny, M.B., Fraser, P., Hyslop, P.S., Selkoe, D.J., and Haass, C. (1996) The Alzheimer's disease-associated presenilins are differentially phosphorylated proteins located predominantly within the endoplasmic reticulum. *Mol. Med.*, **2**, 673–691.

- Weidemann, A., Paliga, K., Durrwang, U., Czech, C., Evin, G., Masters, C.L., and Beyreuther, K. (1997) Formation of stable complexes between two Alzheimer's disease gene products: presenilin-2 and β -amyloid precursor protein. *Nature Medicine*, **3**, 328–332.
- Wong, P.C., Zheng, H., Chen, H., Becher, M.W., Sirinathsinghji, D.J.S., Trumbauer, M.E., Chen, H.Y., Price, D.L., Vander-Ploeg, L.H., and Sisodia, S.S. (1997) Presenilin 1 is required for Notch 1 and DII 1 expression in the paraaxial mesoderm. *Nature*, **387**, 288–292.
- Xia, W., Zhang, J., Perez, R., Koo, E.H., and Selkoe, D.J. (1997) Interaction between amyloid precursor protein and presenilins in mammalian cells: implications for the pathogenesis of Alzheimer disease. *Proc. Natl. Acad. Sci. USA*, **94**, 8208–8213.

14.

PRESENILINS AND THEIR ROLE IN APOPTOSIS

BENJAMIN WOLOZIN AND JAMES PALACINO

Dept. of Pharmacology, Loyola Medical Center, Maywood, IL 60153, USA

OVERVIEW

This chapter focuses on the interaction of presenilins with apoptotic processes. It begins with an overview of the field of apoptosis, covering methods and terminology. We discuss how studies of apoptosis can help us understand the pathophysiology of neurodegenerative diseases and how apoptotic process may actually impact on neurodegenerative illnesses. We will then review research performed implicating presenilins with apoptosis. Wildtype presenilin 2 (PS2) participates enhances apoptosis induced by trophic withdrawal, and mutations in both presenilins sensitize cells to cell death induced by a wide range of factors, including A β , trophic withdrawal, tumor necrosis factor and ceramide. The explanation for this profile of biochemical sensitivity is becoming clearer through analysis of the signal transduction pathways regulated by presenilins. Biochemical studies show that presenilins acts as an endogenous inhibitors of apoptotic cascade. PS2 strongly inhibits both Jun Kinase and NF κ B activity. Loss or gain of activity is problematic for the cells. Overexpression of wildtype PS2 greatly reduces NF κ B activity which removes an important neuroprotective factor from the cell and renders the cell vulnerable to cell death. Mutant forms of PS2 are inactive. This removes an endogenous 'shock absorber' from the cells and increases the cells response to agents that activate the Jun Kinase/NF κ B cascade. This hyper-responsiveness renders the cells vulnerable to stress. Finally, we will relate this work to the larger field of Alzheimer's research and show how the work connecting presenilins with apoptosis may integrate into a broader conceptual model for the pathophysiology of Alzheimer's disease.

INTRODUCTION

The control of cell death is at the heart of medicine (Thompson, 1995). In a myriad of situations, patients' complaints are based on abnormalities in these processes. In Alzheimer's disease the accumulation of β -amyloid (A β) is thought to drive neuronal cell death. Blockage of vasculature leads to stroke and myocardial infarction, which causes oxygen deprivation and cell death. In cancer, the

problem is not enough cell death. The apoptotic machinery prevents cancer by killing cells that are dividing inappropriately, but many cancer cells develop mechanisms to evade apoptosis. Apoptosis is also important for normal body functioning (Jacobson *et al.*, 1997). It shapes our body during fetal development and contributes to normal immune function during adult life. This chapter will focus on the regulation of apoptosis, which appears to be a fundamental part of presenilin biology.

Before delving into the details of presenilins and apoptosis, it is worth pausing and comparing death in biological vs. mechanical systems. Let us examine our cars, for instance. When an engine is deprived of oxygen the engine stops. However, although it is off, the engine is not destroyed. Rather, when the oxygen is restored, the car can start again once the ignition is activated. This is because turning the engine off stops everything in its tracks, without altering the system. Biological systems, though, are different. The equivalent processes, depriving cells of oxygen or telling the cells to 'turn off' does not simply freeze the biochemical processes, rather it changes the cellular metabolism. The cells switch into a metabolic state in which they actively destroy themselves with enzymes such as cysteine directed proteases (caspases), DNA endonucleases and production of free radicals. So, the act of 'turning off' actually 'turns on' processes that fundamentally and irreversibly change the cell.

Throughout this chapter, I will focus on understanding the biochemical processes that are activated during apoptosis. The goal will be to convey an understanding on how the study of cell death is actually a marvelous tool for understanding the biochemical processes that drive both the healthy cell and the dying cell. Apoptosis can be activated by a number of different signal transduction systems. Moreover, there are multiple tools available to carry out the studies, which provides tremendous flexibility in the methods of quantitation and the types of data obtained. Thus, cell death assays can be used to inform us about the signal transduction processes that occur within a cell in response to a stimulus. These are important reasons why the field apoptosis research has exploded—with relative ease and methodological flexibility we gain tremendous insights into metabolic and signal transduction processes within the cell.

Background and Terminology

For a long time, researchers have observed that trophic deprivation leads to rapid death of cells in culture. For instance, in the 1950's Victor Hamburger and Rita Levy-Montalcini commented on how withdrawal of nerve growth factor rapidly induced neuronal death in their superior cervical ganglion cell cultures (Hamburger, 1958). Similarly, developmental biologists have observed the appearance of pyknotic nuclei during development that were interpreted as cell death. In fact, entomologists had long noted that certain cells in insects died at predetermined points in developments and assumed that this cell death was under the control of some kind of master plan (Glucksmann, 1951). However, the field of cell death research did not coalesce until two individuals, a pathologist Richard Wyllie, and a developmental biologist, Roger Horvitz, created the intellectual framework for understanding cell death (Ellis and Horvitz, 1986; Kerr *et al.*, 1972; Wyllie and Kerr, 1980). The importance of this research to human disease received added emphasis with the discovery that one of the regulators of cell death, bcl-2, was also an oncogene (Hengartner and Horvitz, 1994).

Richard Wyllie and his group were studying epithelial cell death in humans and other mammals. Wyllie noted that cell death in the adrenal cortex followed a characteristic pattern of death. The plasma

membrane remained intact, while the chromatin condensed, the nucleus broke up into pieces and the cell ultimately extruded their nuclei (Wyllie and Kerr, 1980). He coined this process, apoptosis (Table 1), which in Greek describes the process of leaves falling from a tree or petals falling from a flower (Wyllie and Kerr, 1980). The other form of cell death, termed necrosis (Table 1), looks quite different. Necrotic cells typically swell up during cell death. Typically, necrosis is due to loss of osmotic integrity, with the nuclei, mitochondria and entire cell lose their compact morphology, swell and sometimes even burst. Wyllie correctly concluded that these two processes, apoptosis and necrosis, were quite different and he went on to show a variety of human disease in which apoptosis played an important role. From the perspective of cell biology distinguishing between apoptosis and necrosis has important implications. Necrosis often results from a traumatic insult and results in nonspecific cell death that is commonly (but not always) unrelated to particular effector pathways. Perhaps the most notable exception to this rule is excitotoxicity which kills cells through a necrotic mechanism but involves specific changes in calcium homeostasis (Ankarcrona *et al.*, 1995). Apoptosis, however, always occurs via activation of particular cell death signal transduction and effector pathways (Martin *et al.*, 1994). Thus, identification of the presence of an apoptotic process implies that particular biochemical pathways are being activated. Conversely, blockade of these processes could stop the cell death process.

Meanwhile, working from the other end of the developmental and scientific spectrum, Roger Horvitz set out to understand the genetics of development by using a very simple system, the small nematode *C. Elegans* (Ellis and Horvitz, 1986). His group mapped the cell fate of every cell in the animal during development and observed that certain cells always died at the same point during nematode development. Like the entomologists that preceeded him, Horvitz correctly assumed that this orchestrated cell death was under genetic control and described it as, *programmed cell death* (PCD). PCD therefore refers to cell death occurring during development (Table 1). Next, Horvitz and colleagues used genetics to identify the genes that control PCD in *C. Elegans*. They found that there were three master genes that regulated cell death, and termed these genes Ced-3, Ced-4 and Ced-9 (Ellis and Horvitz, 1986; Ellis *et al.*, 1991). Ced 3 and Ced-4 are genes that induce cell death (Figure 1), while Ced-9 is a gene that prevents cell death. Loss of Ced-3 or Ced-4 function produced an increase in cell number by preventing PCD in the cells that were destined to undergo die during development (Ellis *et al.*, 1991). Conversely, loss of Ced-9 function reduced cell number by allowing excess PCD (Ellis *et al.*, 1991).

Research over the next decade showed that Ced-3, Ced-4 and Ced-9 all had mammalian homologues. In fact, the cell death systems in mammals turn out to be far more complex than those of *C. Elegans*, and Ced-3, Ced-4 and Ced-9 all correspond with families of homologous genes controlling cell death. The mammalian homologue of Ced-9 is Bcl-2, a mitochondrial protein that controls the ability of mitochondria to buffer cytosolic calcium levels (Hengartner and Horvitz, 1994). As mentioned, there is a family of proteins all of which are homologous to Bcl-2 and act to regulate Bcl-2 function (Yang *et al.*, 1995). Bcl-2 exists as a homodimer with itself or a heterodimer with its homologues. For instance, Bcl-X and Bcl-2 both protect against apoptosis by increasing the ability of mitochondria to lower cytosolic calcium levels, so Bcl-2 homodimers or Bcl-2/Bcl-X heterodimers are cytoprotective (Oltvai *et al.*, 1993; Shimizu *et al.*, 1995). On the other hand, Bak, Bax and Bad both inhibit Bcl-2 function, so Bcl-2/ BAX and Bcl-2/ BAD heterodimers (as well as BAX and BAD

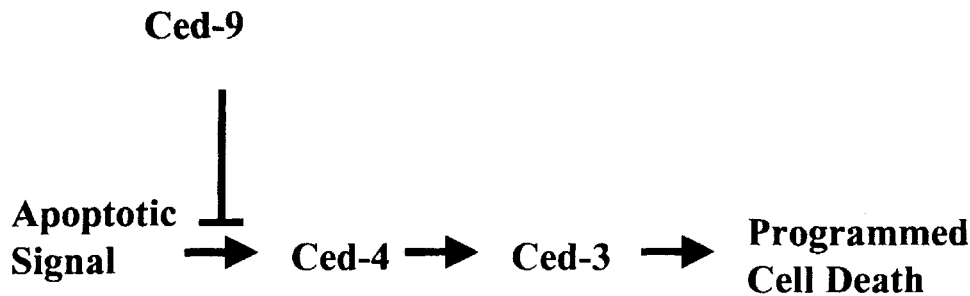


Figure 1. Ced-3/4/9 pathway. The basic machinery producing programmed cell death in the nematode *C. Elegans* consists of three genes. Ced-4 is a protein that activates Ced-3. Ced-4 contains a death domain and is homologous to cytochrome C. Ced-3 is a caspase, also known as a cysteine protease, which carries out the cell suicide program. Ced-9 is homologous to the mammalian gene Bcl-2 and inhibits programmed cell death.

homodimers) are toxic (Chittenden *et al.*, 1995; Oltvai *et al.*, 1993; Yang *et al.*, 1995). Calcium is involved in cell death processes induced by a broad array of agents and conditions. Similarly, Bcl-2 turns out to be cytoprotective in a vast array of different death processes, including apoptosis, necrosis and excitotoxicity. Whenever, calcium, free radicals or mitochondrial function plays an important role in cell death, Bcl-2 is protective (Hockenbery *et al.*, 1993; Lam *et al.*, 1994; Shimizu *et al.*, 1995). Interestingly, Bcl-2 is upregulated in the brains of Alzheimer patients (Su *et al.*, 1997). The neurons that express Bcl-2 show no evidence of DNA fragmentation, suggesting that the Bcl-2 may protect against apoptosis (Su *et al.*, 1997). Bcl-2 is also an oncogene because apoptosis is an important defense that slows down the growth of tumors, thus overproduction or constitutive production of Bcl-2 allows rapid tumor growth and facilitates cancer. Thus, Bcl-2 has profound effects on processes in our body controlling cell proliferation and cell death.

Ced-3 is the executioner, and its mammalian homologues are a class of cysteine directed proteases, termed caspases (Table 1) (Schwartz and Milligan, 1996). The two most well known caspases are interleukin converting enzyme (ICE) and CPP32 (caspase 3), however at least nine different caspases have been identified and more likely exist (Miller, 1997). CPP32 appears to be the most abundant caspase and like Ced-3, loss of CPP32 function has profound developmental effects producing animals with grossly enlarged brains as well as other abnormalities derived from overproduction of cells during development. The biology of caspases is quite complex because they can be activated by a variety of mechanisms and the activation varies depending on cell type (Miller, 1997; Schwartz and Milligan, 1996). Caspases can be alternatively spliced and can exist as inactive proenzymes or as active enzymes (Miller, 1997). For instance, T-cells tend to have caspases present as inactive pro-enzymes. Activation of a death receptor, such as the Fas receptor, produces direct coupling with the pro-enzyme followed by caspase cleavage and activation (Darmon *et al.*, 1995; Enari *et al.*, 1996). Proteins that facilitate the coupling of death receptors with caspases fall into a class of 'death adapter' proteins that includes the nematode protein Ced-4 as well as mammalian proteins such as the recently identified RAIDD protein (Chinnaiyan *et al.*, 1997; Duan and Dixit, 1997; Nagata, 1997; Wu *et al.*, 1997). This allows for particularly rapid activation and rapid apoptosis (occurring within 3 hrs *in vitro*). On the other hand, caspases are not present endogenously in neurons, and are synthesized as active enzymes following an

apoptotic stimulus, such as trophic withdrawal or ischemia (Asahi *et al.*, 1997; Kinoshita *et al.*, 1997). Caspases have a variety of substrates, all of which are somehow intimately involved with apoptosis. These substrates include poly-adenosine-ribose polymerase, actin, PAK and, interestingly, both presenilin 1 and presenilin 2 (Kayalar *et al.*, 1997; Kim *et al.*, 1997b; Lazebnik *et al.*, 1994; Mashima *et al.*, 1995; Rudel and Bokoch, 1997). The regulation of PS1 and PS2 cleavage by caspase will be discussed in more detail below, but is an important piece of evidence supporting the role of presenilins in apoptosis.

Methods Used in Apoptosis Research

Part of the power of apoptosis research lies in the ease of identifying cells undergoing apoptosis and the great ability to quantitate the phenomenon. At this point in the development of the field, the 'classic' methods for detecting apoptosis revolve around the DNA degradation that occurs during apoptosis. During apoptosis the nucleus fragments and the DNA is cleaved by a DNA endonuclease into oligomeric units of ~250 base pairs that correspond with the distance between nucleosomes. Both of these processes, nuclear fragmentation and DNA oligomerization, occur late in the apoptotic process but distinguish apoptosis from necrosis (Darzynkiewicz *et al.*, 1994; Gavrieli *et al.*, 1992; Wood *et al.*, 1993). Necrotic nuclei enlarge rather than fragment and necrotic DNA is broken up randomly to produce a smear of DNA fragments (Darzynkiewicz *et al.*, 1994). The smear occurs because free radicals cause the DNA fragmentation during necrosis and the free radicals cleave the DNA randomly independent of the position of the nucleosomes. There are three important that use the nuclear changes to identify apoptotic cells and distinguish apoptosis from necrosis. The first, and easiest, is to use DNA stains, such as propidium iodide, Hoechst 33258, Hoechst 33342 or ethidium bromide, to label nuclei (Darzynkiewicz *et al.*, 1994). These stains can be analyzed by microscopy or by fluorescent activated cell sorting. A cell sorter will quantitate the amount of DNA, so that healthy cells have a diploid or tetraploid amount of DNA, depending on their point in the cell cycle, while apoptotic and necrotic cells will have less DNA. Similarly, by microscopy healthy nuclei are round and of average size (8–12 μ M), necrotic nuclei are round and distended and late stage apoptotic nuclei are fragmented into 3–5 smaller round pieces (Table 2). A second method is to isolate apoptotic DNA and analyze it by gel electrophoresis. The isolation is facilitated by the fact that apoptotic DNA is fragmented and can be obtained by permeabilizing the cell with a weak detergent such as Triton X-100, whereas genomic DNA is largely insoluble and remains in the nucleus (Darzynkiewicz *et al.*, 1994). After isolation with such procedures, genomic DNA will be absent, necrotic DNA will give a broad smear pattern, while apoptotic DNA will give a characteristic 'DNA ladder' corresponding to DNA bands appearing in increments of ~250 bases. The most widely used method, however, is called terminal UTP-Nick End Labeling (TUNEL) (Gavrieli *et al.*, 1992). This is a very sensitive method that is easy and the best way of identifying a small number of apoptotic cells (perhaps too few to provide DNA for gel analysis) *in vivo*. Normal human DNA has 46 chromosomes and therefore 92 DNA ends. However, apoptotic DNA has been fragmented and therefore has many, many free ends. The TUNEL method used DNA labeling enzymes, such as terminal deoxynucleotidyl transferase or the Klenow fragment of DNA polymerase to add biotinylated nucleotides onto the ends of DNA (Wood *et al.*, 1993). DNA ends can then be labeled using streptavidin-coupled peroxidase and identified with a di-

amino-benzidine-mediated colorimetric reaction. Healthy DNA will have a low number of biotinylated nucleotides attached and won't show up with the colorimetric reaction, whereas apoptotic nuclei will stain darkly brown. A weakness of this method is that necrotic DNA will also stain, however time course analyses have shown that apoptotic nuclei become TUNEL positive much earlier in the cell death process than do necrotic nuclei (Gold *et al.*, 1994). This weakness is compensated by the exquisite sensitivity of the TUNEL method. This is particularly relevant to *in vivo* work where the TUNEL method is uniquely able to detect rare apoptotic events among a large number of normal cells. Moreover, when interpreted judiciously and performed with appropriate negative controls the method can be used with a good deal of confidence and is therefore very powerful.

An increasing number of other methods are also being used to identify apoptosis, and I will cover some of the more common methods because they are so useful. As DNA fragments, histones are released into the cytoplasm; based on this, measurements of soluble histone have been adapted to quantitate apoptosis. One useful assay is FITC-coupled Annexin-V which detects changes in the plasma membrane of apoptotic cells that occur early (minutes to hours) in the apoptotic process (Vermes *et al.*, 1995). Annexin-V binds phosphatidyl serine, a lipid that normally faces the cytoplasm but during apoptosis also faces the extracellular space. A useful aspect of this assay is that when combined with propidium iodide, which is excluded from apoptotic cells but not from necrotic cells, the assay distinguishes apoptosis from necrosis. During apoptosis there is a profound decrease in transcription and translation of molecules unrelated to apoptosis. Measurement of activity of a 'constitutively' expressed reporter gene, such as CMV- β -galactosidase or CMV-luciferase vectors, can be used to quantitate this decrease in transcriptional/translational activity (Vito *et al.*, 1996b). Finally, several assays measure changes in mitochondrial function associated with cell death. The (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay measures changes in redox potential that occurs during cell death and is very sensitive to agents such as A β (Shearman *et al.*, 1994). Dichlorofluorescein diacetate measures hydrogen peroxide and superoxide production in the mitochondria, while luciferase assays can be modified to measure ATP levels in mitochondria. Cytochrome-C is normally found attached to the mitochondria, but release of the protein into the cytoplasm may be one of the first steps in the apoptotic process (Kluck *et al.*, 1997). Thus, multiple assays are available to measure cell death.

Several questions arise when debating which assay to use. A basic question is whether one is working with tissue sections or cells in culture. Very few methods are useful in tissue sections; TUNEL is really the mainstay. On the other hand, virtually all the methods are applicable to cell culture. Another important question is whether one cares if the mode of death is apoptotic or necrotic. If the distinction is important, then the choice of assays is more limited. Frequently, a good strategy is to begin by proving the mode of cell death, using DNA fragmentation, TUNEL or Annexin-V, and then to progress onward to easier or less expensive assays. Having established the mode of cell death, one then tailors the assays. Some assays, such as the MTT or luciferase/ATP assays start with a high basal activity that decreases with cell death; such assays are useful when most cells are dying but not useful when only a small number of cells are dying, such as during transient transfections. On the other hand, Annexin-V, CMV- β -gal and N, N-dichlorofluorescein are all highly adaptable to situations like transient transfections where only a small number of cells are dying. Thus, there is tremendous flexibility in choice of methods for study of apoptosis.

PRESENILIN BIOLOGY

Presenilins: A Role for PS2 in Apoptosis

With this methodologic primer as a background it is now possible to begin to assess what has been accomplished in the area of presenilins and apoptosis and what the significance of these results are for Alzheimer research.

Presenilins are the genes that cause familial AD. Familial AD is transmitted as an autosomal dominant disorder and is characterized by an age of onset that is generally below age 65. Most cases of familial AD are caused by Presenilin 1 (PS1), however there are two pedigrees, one from Germany and one from Italy in which AD is caused by a mutation in a homologous gene, termed Presenilin 2 (PS2) (Levy-Lahad *et al.*, 1995; Sherrington *et al.*, 1995). PS1 and PS2 code for 46 and 55 KDa proteins, respectively, that are about 80% homologous and appear to have eight transmembrane domains (Figure 2) (Doan *et al.*, 1996; Levy-Lahad *et al.*, 1995). Although PS1 and PS2 are synthesized as single peptide holoproteins, they are actually rapidly cleaved and exist endogenously as the smaller cleaved proteins. The cleavage site is just after the 6th transmembrane domain (Figure 2). For PS1, the cleaved peptides have sizes of 17 and 29 KDa for the N and C terminal peptides, respectively, while the PS2 cleavage fragments are 19 and 38 kDa, respectively (Kim *et al.*, 1997a; Thinakaran *et al.*, 1996). Interestingly, the membrane spanned by the presenilins appears to that of the endoplasmic reticulum (ER), not the plasma membrane (Elder *et al.*, 1996; Kovacs *et al.*, 1996). Consistent with the ER localization, immunohistochemical studies indicate that PS1 is expressed most abundantly in dendrites of neurons (Elder *et al.*, 1996).

The localization of PS1 and PS2 to the endoplasmic reticulum provides an intriguing clue about function. The endoplasmic reticulum is one of the key organelles involved in processing proteins and is the first destination for all proteins destined for the Golgi, lysosome or plasma membranes. This observation has lead to the proposal that presenilins play an important role in membrane trafficking (Weidemann *et al.*, 1997). Since amyloid precursor protein (APP) passes through the endoplasmic reticulum, one proposed function for presenilins is in the regulation of trafficking of proteins such APP (Weidemann *et al.*, 1997). In this scenario, mutations alter presenilins function, which changes APP trafficking and leads to abnormal production of A β ₁₋₄₂. The recent findings that PS1 and PS2 do associate with APP provides support for a model in which presenilins directly bind APP and affect APP processing by regulating protein trafficking (Weidemann *et al.*, 1997; Xia *et al.*, 1997). To date, no mutation-related changes in the presenilin/APP have been identified to date, which raises questions about whether the trafficking model actually accounts for the changes in production of A β ₁₋₄₂. However, the absence of mutation-related affects of the PS1/APP interaction may change as more is known.

The endoplasmic reticulum has other functions besides protein trafficking. It plays an important role in signal transduction, containing G-proteins, receptors for inositol phosphates and calcium channels. Since the endoplasmic reticulum can have a profound in-fluence on cytosolic calcium levels, it also plays an important role in regulating apoptosis. Thus, the localization of the presenilins to the endoplasmic reticulum provides one of the first clues that these proteins could influence apoptosis.

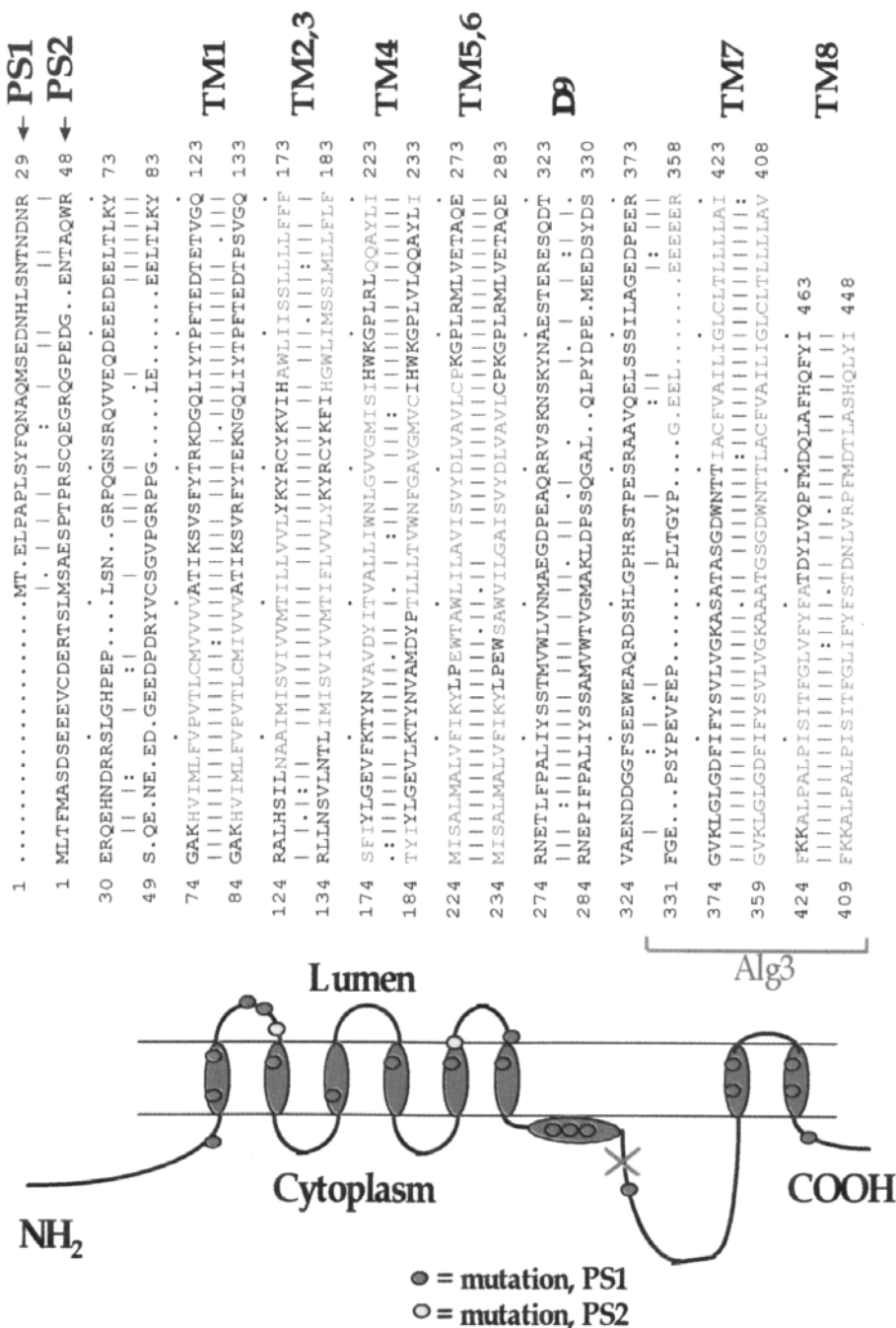


Figure 2. PS1 and PS2 structure. PS1 and PS2 are homologues. This diagram shows the protein alignment of PS1 (upper strand) and PS2 (lower strand). The transmembrane (TM) domains are shown in green, and the domain after TM6 lying next to the membrane is shown in blue. The region of PS2 corresponding to Alg-3 is shown in rose. A graphical representation of PS1 is shown alongside the sequence. The TM domains in the graphical representation are lined up with those of the amino acid sequence. Some of the known Alzheimer related mutations are shown for both PS1 (red dots) and PS2 (yellow dots). (See Colour Plate V)

The connection between presenilins and apoptosis turns out to be an exceedingly strong connection. The first evidence for this came from studies by D'Adamio, Vito and colleagues who were cloning genes that could prevent Fas-mediated apoptosis in the T-cell line, 3DO (Vito *et al.*, 1996a). They transfected in an episomal cDNA library from activated T-cells and saved cells that did not die in response to activation by Fas ligand. The process was repeated several times after which they isolated the episomal cDNAs that were in the death resistant cells (Vito *et al.*, 1996a). One of these genes, which they termed Alg-3, coded for the C-terminal sequence of Presenilin 2, PS2₃₄₆₋₄₄₈ (Figure 2). CDO cells expressing Alg-3, were resistant to cell death induced by Fas ligand, staurosporine or C2-ceramide. This data provided an important clue that PS2 could regulate apoptosis, however it also left open many questions. Alg-3 is not a naturally occurring protein and 3DO cells are not neurons.

Further work, based on a collaboration between members of my laboratory and D'Adamio's laboratory, clarified many of these issues and established a strong link between presenilins and apoptosis (Vito *et al.*, 1996b; Wolozin *et al.*, 1996). Using differentiated PC12 cells as a model system, we were able to show that full length PS2 does indeed play an important role in apoptosis, and it does so in neurons as well as T-cells. We found that differentiated PC12 cells transfected with full length PS2 showed enhanced apoptosis in response to trophic withdrawal compared to cells transfected with vector (pcDNA3) alone (Figure 3) (Wolozin *et al.*, 1996). These effects were evident both with the TUNEL method and with DNA fragmentation gel analyses. The TUNEL studies provided the quantitation, while the observation of a DNA ladder with gel electrophoresis distinguish apoptosis from necrosis and prove that the system is following an apoptotic pathway. Although, this data indicates that PS2 can affect cell toxicity, taken alone it does not actually prove that PS2 is involved in apoptosis because expression of any toxic protein would be expected to enhance apoptosis. It is conceivable that overexpression of any protein in the endoplasmic reticulum might affect apoptosis. However, transfection with a truncated form of the IP3 receptor that localizes to the endoplasmic reticulum (a gift from G.Mignery, Loyola University) does not affect apoptosis (Alexander *et al.*, 1997). This suggests that nonspecific toxicity is probably not a factor in these assays.

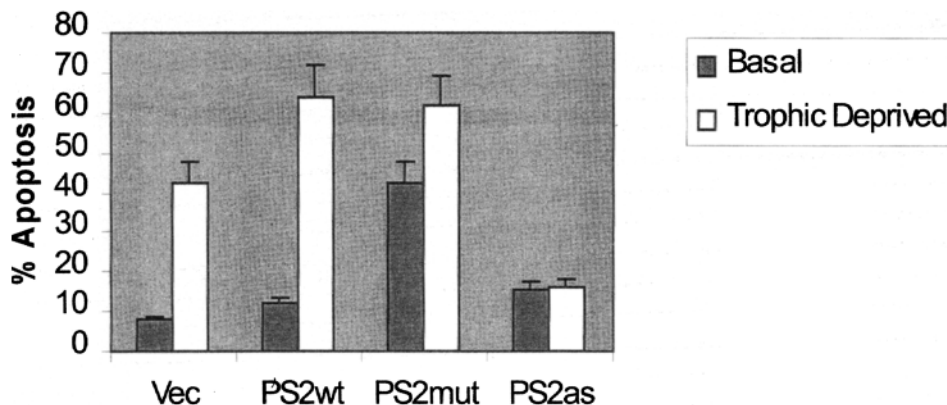


Figure 3. PS2/PC12 transfection. Transfection of the N141 PS2 into NGF differentiated PC12 cells increases apoptosis under basal condition. Transfection of both wildtype PS2 (PS2wt) and mutant PS2 (PS2mut) increases apoptosis during trophic withdrawal, while transfection of antisense PS2 (PS2as) inhibits apoptosis under trophic withdrawal.

The more important experiment in these types of studies is to examine the effects of blockade of endogenous PS2 activity. The idea is that if an endogenous protein contributes to the apoptotic process, then removal of this protein should decrease or delay apoptosis by impairing the process. This type of strategy has been used with great efficacy in multiple systems to indicate an essential role for a particular protein in a particular process. For instance, the original identification of the genes regulating apoptosis in *C. elegans* was based on observation of the effects of loss of function on cell number. In order to show that PS2 was part of the actual machinery that carries out apoptosis, we investigated the effects of reducing PS2 protein levels by transfecting in vectors containing antisense PS2. Immunoblots showed that antisense PS2 effectively reduced PS2 protein levels, while electrophoretic analysis of DNA fragmentation and TUNEL analyses both showed that antisense PS2 constructs protected against apoptosis induced by trophic withdrawal (Figure 3) (Wolozin *et al.*, 1996). The fact that reduced levels of PS2 decrease or delay apoptosis indicates that endogenous PS2 is playing an active role in the apoptotic process. Knowing that native PS2 enhances apoptosis provides perspective with which we can begin to understand the action of Alg-3, the short cDNA coding for the C-terminal region of PS2. Alg-3 also inhibited apoptosis induced by trophic withdrawal of PC12 cells. This suggests that Alg-3 interferes with the function of PS2. Taken as a whole the data indicate that PS2 is part of the biochemical machinery that carries out apoptosis, and removal of PS2 or inhibition of PS2 function blocks the apoptotic process.

Presenilin 1 also Enhances Apoptosis

Based upon the high homology between PS1 and PS2 (Figure 2), one would predict that the proteins have related functions. Indeed, studies by Mattson's group have shown that PS1 also enhances apoptosis (Guo *et al.*, 1996; Guo *et al.*, 1997). They generated cell lines of PC12 cells that stably express PS1. These cell lines showed enhanced cell death in response to trophic withdrawal or β -amyloid. Unfortunately, unlike PS2, the antisense PS1 transcript does not reduce PS1 levels so it is not possible to use this strategy to test the effects of removal of endogenous PS1. However, as with the PS2 work, the mode of cell death suggests apoptosis. Hoechst dye staining reveals nuclear fragmentation, which is characteristic of apoptosis, and the protein synthesis inhibitor cycloheximide reduces cell death, indicating that new synthesis of pro-apoptotic proteins (such as caspases) are necessary for the cell death. Guo, Mattson and colleagues have also taken this work a step further by showing that antioxidants and Bcl-2 protect against the PS1-related apoptosis, suggesting that hydrogen peroxide and superoxide free radicals may be involved in the process (Guo *et al.*, 1996; Guo *et al.*, 1997). Thus, PS1 appears to have effects on apoptosis similar to those of PS2.

Presenilins as Substrates for Caspases

Studies on protein processing provide further evidence implicating PS2 in apoptosis. As mentioned above PS1 and PS2 are synthesized as single peptide holoproteins, they are rapidly cleaved and exist endogenously as the smaller cleaved proteins. The cleavage site for this constitutive processing is at the beginning of the large cytoplasmic loop after transmembrane domain 6, which yields a 25 kDa C-terminal PS2 cleavage fragment and a 17 kDa C-terminal PS1 cleavage fragment. However, during

apoptosis, both PS1 and PS2 become substrates for caspases yielding smaller C-terminal fragments (Kim *et al.*, 1997b). For PS2, the cleavage occurs at residue 330, which produces a 20 kDa C-terminal cleavage fragment that is very similar to the protein produced by Alg-3. A similar pattern is seen for PS1, yielding a 14 kDa C-terminal fragment. Like Alg-3, the 20 kDa caspase cleavage fragment of PS2 is anti-apoptotic and interferes with the actions of PS2. The targeting of the presenilins by caspases strengthens the connection between presenilins and apoptotic pathways. In addition, the observation that the C-terminal caspase cleavage fragment is anti-apoptotic suggests a mechanism for feedback inhibition of presenilin action.

Signal Transduction Pathways Controlling Apoptosis

Before we can commence with a discussion of how presenilins regulate apoptosis, I will digress and provide some background information on the signal transduction pathways controlling apoptosis. Apoptosis can be divided into three phases. The first phase encompasses initiation. Apoptosis can be initiated either by activating a death receptor or by damaging the cell. The family of receptors termed, death receptors, include the Fas receptor, the TNF α type I receptor and the p75 Nerve Growth Factor receptor, but there are also likely to be other homologous receptors (Nagata, 1997). The structure of these receptors is known best for the TNF α receptor and the Fas receptor, but is likely to be similar for the other receptors. These receptors have a characteristic structure with several different domains (Figure 4). One domain is termed a 'death domain'. This domain binds a death

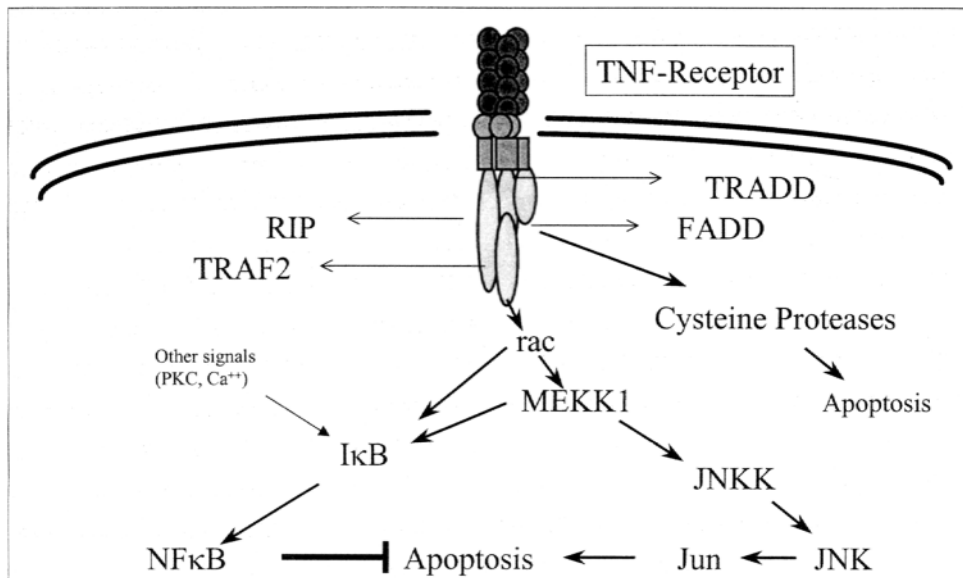


Figure 4. TNF α receptor 1I and signal transduction pathway. The apoptotic pathway mediated by c-Jun Kinase is a multistep pathway. NF κ B is often activated in parallel with JNK. Whether the TNF α receptor initiates apoptosis via the JNK cascade or via a death domain coupled mechanism depends on the cell type.

adapter protein that can directly couple to caspases and activate them without any apparent requirement for second messengers (Duan and Dixit, 1997; Nagata, 1997). Cells that are prepared for apoptosis, such as T-cells, use this pathway to rapidly activate caspases; thus, T-cell lines undergo apoptosis within three hours of receptor engagement (Enari *et al.*, 1996). However, many cells, such as neurons, do not have caspases present normally. These cells activate apoptosis through a different domain that activates a signal transduction cascade and requires gene transcription and protein synthesis (Figure 4) (Estus *et al.*, 1994; Verheij *et al.*, 1996). Finally, death receptors have a third domain that activates a signal transduction pathway that includes the enzymes TRADD and TRAF, which is unrelated to cell death (Liu *et al.*, 1996; Tewari and Dixit, 1996).

The second phase of apoptosis is the signal transduction phase, in which second messengers activate the biochemical machinery that carries out the apoptotic process during the third phase, the execution phase. The signal transduction systems controlling apoptosis proceed through several separate pathways. Although there is likely to be cross talk between these pathways, the research into this field has not yet evolved to a stage in which the cross talk has been addressed. The main signal transduction cascades controlling apoptosis are the Jun Kinase cascade, the protein kinase B cascade and the p53 cascade (Caelles *et al.*, 1994; Kaufmann-Zeh *et al.*, 1997; Verheij *et al.*, 1996). Activation of apoptosis through the JNK cascade appears to occur via the interaction of the small GTP binding protein, rac, with the death receptors. Rac then activates a sequential series of kinases that includes MEKK1, JNKK, p38, jun kinase and ultimately leads to activation of the transcription factor c-Jun (Figure 4) (Johnson and Vaillancourt, 1994). This pathway can be confusing because receptor tyrosine kinases can also activate the JNK cascade, but they do so in conjunction with activation of the pro-growth pathway mediated by ERK (Cobb *et al.*, 1991; Crespo *et al.*, 1994; Xia *et al.*, 1995). Activation of the JNK and ERK cascades in tandem does not appear to stimulate apoptosis, while activation of the JNK cascade alone does stimulate apoptosis. The JNK cascade though clearly is important for apoptosis because, expression of dominant negative JNK or c-Jun constructs both can prevent apoptosis mediated by trophic withdrawal (Verheij *et al.*, 1996; Xia *et al.*, 1995).

A second cascade that can stimulate apoptosis is a cascade mediated by a kinase termed protein kinase B (PKB, also known as Akt) (Dudek *et al.*, 1997; Kohn *et al.*, 1996). This pathway is unusual in that many of the proteins in the pathway are negative regulators (Figure 5). The PKB pathway begins with receptor engagement of the enzyme phosphatidyl inositol 3-kinase, which stimulates PKB activity (Franke *et al.*, 1995; Kennedy *et al.*, 1997). Activation of PKB stimulates the S6 kinase and the transcription factors FRAP and myc but inhibits glycogen synthase kinase 3 (GSK-3) as well as caspases (Hemmings, 1997). Since GSK3 and caspases stimulate apoptosis, activation of PKB inhibits apoptosis, while inhibition of PKB activates apoptosis. DNA damaging agents (such as etoposide) and chemicals that inhibit PI3 kinase (such as wortmannin), appear to activate the signal transduction pathway that involves this PI3 kinase, PKB pathway (Kaufmann-Zeh *et al.*, 1997). Interestingly, GSK3 β has also been shown to phosphorylate tau and may be important to inducing formation of neurofibrillary tangles (Takashima *et al.*, 1996). A third cascade that may control apoptosis is one mediated by p53, and is intimately connected with control of the cell cycle. This pathway is also activated by DNA damage, which activates p53 and causes the cell to stop the cell cycle progression (Sakhi *et al.*, 1994). However, the mechanism by which this interruption of the cell cycle causes apoptosis is not currently known.

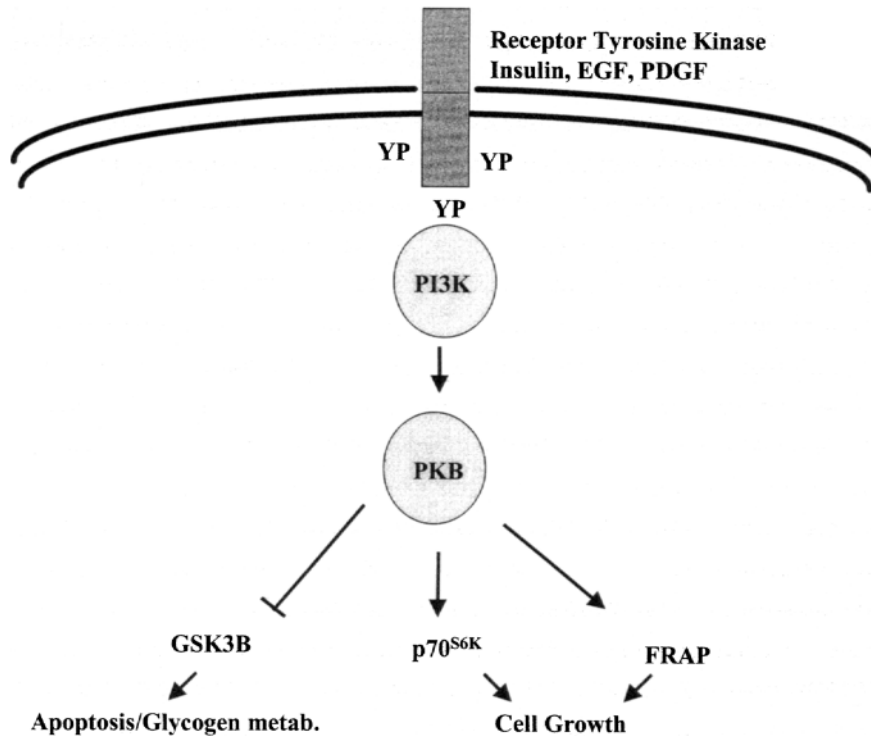


Figure 5. PKB apoptotic pathway. Activation of receptor tyrosine kinases leads to autophosphorylation and engagement of PI3 kinase (PI3K, consisting of a p85 and a p10 subunit). Activated PI3 kinase stimulates PKB (also known as Akt) which acts on a variety of different proteins. Among the proteins regulated by PKB is GSK3 β , which is inhibited by PKB and is capable of phosphorylating the microtubule associated protein tau. Loss of PKB activity leads to activation of GSK3 β and apoptosis.

Integrating the actions of these three pathways leaves us with a picture of three pathways that can stimulate apoptosis, the death adapter pathway, the JNK cascade and the p53 pathway, and one pathway, the PKB pathway whose tonic activity is necessary to inhibit apoptosis. However, the apoptotic cycle is not the normal state of existence for a cell, and so perhaps it should come as no surprise that there are multiple systems in place to inhibit apoptosis. One very important inhibitor of apoptosis is Bcl-2 and its homologues, which was discussed earlier in this chapter (Hockenbery *et al.*, 1993). Bcl-2 functions as a dimeric protein and appears to act as a mitochondrial calcium channel that helps the mitochondria to absorb cytosolic calcium, which can activate apoptosis at high cytosolic concentration (Lam *et al.*, 1994). Bcl-2 may also inhibit apoptosis by preventing the transfer of cytochrome C from the mitochondrial membrane to the cytosol; soluble cytochrome C appears to be one of the messengers that signals caspase activation, although the mechanism is unknown (Kluck *et al.*, 1997). The buffering of calcium and stabilization of cytochrome C also appears to reduce mitochondrial free radical production, perhaps by keeping the electron transport system intact. The regulation of Bcl-2 is quite interesting because Bcl-2 can exist as a homodimer with itself or a

heterodimer with its homologues. The Bcl-2 homodimer and the Bcl-X/Bcl-2 heterodimers are both active and both protect against apoptosis (Shimizu *et al.*, 1995). Conversely, BAX and BAD both inhibit Bcl-2 function, so Bcl-2/ BAX and Bcl-2/ BAD heterodimers (as well as BAX and BAD homodimers) promote apoptosis (Chittenden *et al.*, 1995; Oltvai *et al.*, 1993; Yang *et al.*, 1995).

A second protein that appears to inhibit apoptosis is the transcription factor NFκB. The role of NFκB in cell function though appears to be more complicated than that of Bcl-2. NFκB was originally identified in 1988 by Baltimore and Sen as a transcription factor that is present in T-cells and is necessary for immune cell activation *in vivo* (Sen and Baltimore, 1986). NFκB turns out to be ubiquitously present and regulates transcription of multiple genes including genes relevant to AD such as the cytokine IL6, the RAGE receptor, which binds Aβ, and the master developmental neuronal switch, NOTCH (Baldwin, 1996). For several years it has been noted that NFκB becomes active during cell stress situations, so for instance, NFκB is a good marker for Aβ toxicity *in vitro*. Recently, however several groups noted that overexpression of NFκB actually protects cells against apoptosis (Beg and Baltimore, 1996; Van Antwerp *et al.*, 1996; Wang *et al.*, 1996). Thus, NFκB appears to lead a somewhat schizophrenic existence in which its actions may vary greatly depending on the cell type and situation. NFκB induces the transcription of genes necessary for immune activation, but in non-immune cells NFκB may induce synthesis of anti-apoptotic genes.

NFκB is a cytoplasmic protein made up of two subunits, c-rel (also known as p65) and p50 (Baldwin, 1996). C-rel is actually part of a family of homologous proteins all of which can substitute in NFκB; thus, the composition of the NFκB dimer varies depending on cell type. Recent studies have shown that NFκB is activated in tandem with the JNK pathway by a variety of stimuli including tumor necrosis factor (TNFα), ultraviolet light or hydrogen peroxide (Baldwin, 1996; Lee *et al.*, 1997). Activation of NFκB appears to modulate the apoptotic potential of the JNK cascade, because overexpression of the p65 and p50 together blocks apoptosis. Thus, the activation of the JNK cascade induces apoptosis in absence of NFκB activation, but will not induce apoptosis in the presence high levels of active NFκB (Van Antwerp *et al.*, 1996; Wang *et al.*, 1996). Since many of the stimuli that activate NFκB also activate the JNK cascade, the two pathways are presumed to be linked, and the linkage appears to be through the kinase MEKK1, which is a component of the JNK cascade but can also activate NFκB (Figure 4). In order to understand how MEKK1 works, though, we first need to talk about the protein that is most directly responsible for regulating NFκB function, IκB. The IκB protein is an inhibitor of NFκB that directly binds NFκB and masks a site controlling nuclear translocation (Verma *et al.*, 1995). This sequesters the IκB/NFκB complex in the cytoplasm. Phosphorylation of IκB releases it from NFκB, which unmask the nuclear translocation site and allows NFκB to rapidly translocate to the nucleus (Alkalay *et al.*, 1995). Once in the nucleus, NFκB acts as a transcription factor inducing the transcription of multiple genes including some that are anti-apoptotic (Beg and Baltimore, 1996; Lee *et al.*, 1997).

The details of IκB regulation are only beginning to be understood. Like c-rel, there are several IκB homologues, which indicates that there is likely to be cell type variation in control of IκB. Regulation of NFκB predominantly occurs by modulating IκB phosphorylation. Once phosphorylated and released from NFκB, IκB is ubiquitinated and rapidly degraded by the proteasome (Baldwin, 1996; Lee *et al.*, 1997). Several signals affect IκB phosphorylation. Protein kinase C can act together with calcium to stimulate IκB phosphorylation. MEKK1, a kinase in the JNK cascade, also stimulates IκB

phosphorylation as well as stimulating JNK activity. Finally, a recently cloned kinase, JIF1, appears to be the first kinase identified whose function is specific to the I κ B/NF κ B cascade. Thus, multiple discrete pathways stimulate I κ B phosphorylation.

Biochemical Pathways Sensitive to PS1 and PS2

Based on this knowledge of the signal transduction cascades we can now begin to understand how presenilins might influence apoptosis. The first indications of which biochemical pathways are regulated by presenilins comes from an analysis of what chemicals can potentiate presenilin toxicity. The initial work describing the cloning of Alg-3 showed that Alg-3 inhibits apoptosis induced by staurosporine or C₂-ceramide, but not by dexamethasone or actinomycin D (Vito *et al.*, 1996a). Alg-3 and antisense PS2 also inhibit apoptosis due to oxidative stress induced by glutamate (Vito *et al.*, 1996b). PC12 cells are killed by glutamate through blockade of cysteine transport, which decreases glutathione levels and increases oxidative stress. On the converse side, overexpression of PS2 enhances apoptosis mediated by staurosporine, hydrogen peroxide or β -amyloid (Deng *et al.*, 1996; Wolozin *et al.*, 1996). In my laboratory we have also observed that both PS1 and PS2 also

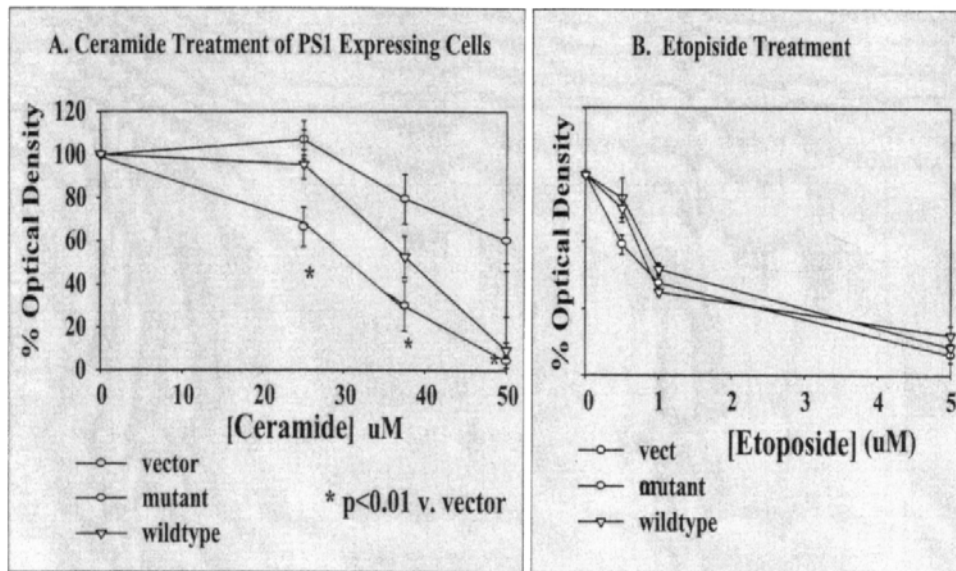


Figure 6. Mutant PS1 increases the sensitivity of Jurkat cells to C₂-ceramide. Jurkat cells were generated inducibly expressing wildtype or mutant (H115Y) PS1 under control of the ecdysone promoter. Cells expressing mutant PS1 are much more sensitive to toxicity due to the sphingolipid, C₂-ceramide, which activates the JNK cascade, than to etoposide, which induces DNA damage.

enhance apoptosis mediated by TNF α or C₂-ceramide, but do not enhance apoptosis due to etoposide (Figure 6). Finally, as mentioned above, overexpression of PS1 also enhances apoptosis mediated by hydrogen peroxide and β -amyloid.

These disparate chemicals fall into distinct groups based upon the mechanism by which they induce apoptosis. For instance, β -amyloid is thought to kill cells via production of hydrogen peroxide (Behl *et al.*, 1994). Thus, hydrogen peroxide and β -amyloid act via a common pathway. Studies on the mechanism of action of hydrogen peroxide, in turn, suggest that it acts by activating an apoptotic cascade involving Jun Kinase (JNK) and NF κ B (Baldwin, 1996). TNF α , the second messenger C₂-ceramide and oxidative stress also activate the JNK/NF κ B cascade (Verheij *et al.*, 1996). Thus, five of the six agents that are sensitive to presenilin overexpression act via the JNK/NF κ B cascade. Staurosporine, the sixth agent, inhibits protein kinase C activity. While this can be related to the JNK/NF κ B cascade, it is unclear whether the JNK/NF κ B cascade is the main target of staurosporine-mediated apoptosis. What of the agents that are insensitive to presenilin overexpression, such as dexamethasone, etoposide or actinomycin D? The first two of these agents, dexamethasone and etoposide appear to kill cells via the PKB pathway (Kauffmann-Zeh *et al.*, 1997). Thus, synthesis of this information suggests that the interaction of presenilins with the JNK/NF κ B cascade is probably important.

Presenilins Regulate the JNK/NF κ B Cascade

In order to determine the mechanism by which presenilins regulate apoptosis, we have begun to directly examine the regulation of JNK/NF κ B by presenilins. Our studies of PS2 are further along than the studies of PS1, so I will focus on PS2 first, however, the preliminary information for PS1 shows some similarities to the actions of PS2.

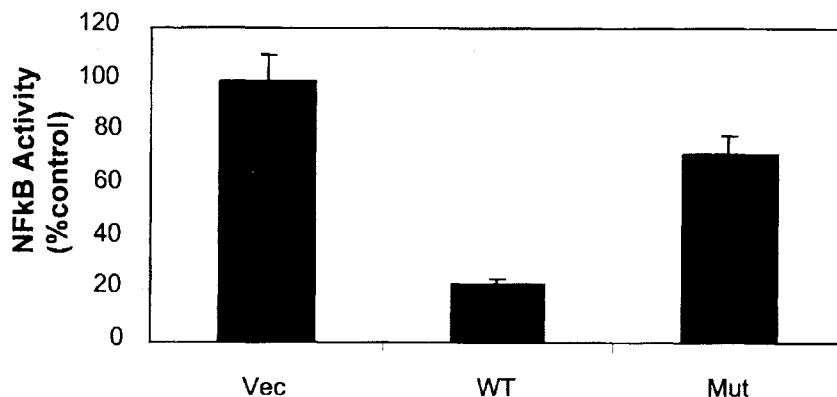


Figure 7. PS2/NF κ B effects. Cotransfection of wildtype PS2 (WT) with a luciferase reporter vector reduces basal or stimulated activity of NF κ B (basal activity shown). The N1411 mutant of PS2 (mut), however, shows much less inhibitory activity in PC 12 cells.

The studies with PS2-transfected cells, in which the PS2 contains an N-terminal FLAG tag, indicate that wildtype PS2 inhibits the NF κ B/JNK cascade (Alexander *et al.*, 1997). One technique that has been exceedingly useful in examining presenilin function has been the use of luciferase-based reporter vectors. In order to examine the regulation of NF κ B by PS2, we transfect PS2-overexpressing cell lines with a luciferase reporter gene driven by a DNA sequence corresponding for the consensus

sequence of the NF κ B promoter or the AP1 promoter. Cells have a basal level of NF κ B or JNK activity, which can bind to the NF κ B enhancer, or for JNK activate c-Jun and stimulate the AP1 enhancer, and drive production of luciferase, whose enzyme activity is detected spectrophotometrically following reaction with appropriate substrates. Activation of NF κ B or JNK increases the amount of luciferase, while inhibition results in less basal luciferase activity (or less stimulated activity compared to a control cell line). Using these assays, we have observed a striking 80–90% reduction of basal and stimulated NF κ B activity in cell lines overexpressing FLAG-tagged wildtype PS2 (Figure 7). A similar decrease is seen in JNK driven activity. Inhibition is seen following stimulation NF κ B by different mechanisms, such as tumor necrosis factor α (TNF α) or a combination of the protein kinase C agonist Phorbol ester dibutyrate and the calcium ionophore ionomycin. These results indicate that overexpression of the FLAG-PS2 construct inhibits the JNK/NF κ B cascade.

Even endogenous PS2 appears to exert tonic inhibition over the JNK/NF κ B cascade. Transfection of cells with constructs that can inhibit endogenous PS2 function, one construct coding for anti-sense PS2 and the other coding for Alg-3, increases NF κ B activity by about 70%, suggesting that the constructs block tonic inhibition by endogenous PS2. The NF κ B assay also provides a good method for performing structure function analyses on PS2. We obtained a panel of PS2 C-terminal deletion mutants as well as chimeric PS1/PS2 genes from Christian Haas, and investigated the affects of these constructs on NF κ B function. Transfection of cells with PS2 constructs deleted prior to the 7th transmembrane domain or use of the chimeric constructs increased NF κ B activity and protected against apoptosis, suggesting that they were interfering with PS2 activity. These data suggest that the active species of PS2 is the holo-protein and that the cleaved forms of the molecule are inactive.

Our studies of PS1, although incomplete, show a smaller effect on the NF κ B/JNK pathway. PS1 inhibits the NF κ B by only 40% and also appears to have a smaller affect on JNK than PS2. The anemic affect of PS1 suggests that it is probably not directly regulating the JNK/NF κ B pathway. The enhanced vulnerability of cells expressing mutant PS1 is therefore likely to be due to effects on an alternate apoptotic cascade, such as the Akt/GSK3 β cascade.

Since many of the enzymes in the JNK and NF κ B cascades have been identified and cloned, it is possible to determine the entry point of PS2 into these cascades. For instance, the enzyme MEKK is known to activate NF κ B by stimulating the phosphorylation of I κ B (Lee *et al.*, 1997). However, PS2 is able to block MEKK activation of NF κ B, which indicates that PS2 is acting downstream of MEKK. This blockade appears to be at or above the level of I κ B because cells overexpressing PS2 show no changes in I κ B levels in response to a TNF α challenge. The ability of PS2 to block changes in I κ B indicate that it is blocking or inactivating the signals to I κ B, which shows that PS2 acts proximal to this protein. The mechanism of action of PS2 remains unclear, but the point of regulation in the signal transduction cascade of PS2 is clear (Figure 7); PS2 acts between MEKK and I κ B to inhibit the NF κ B/JNK cascade. Taken together, these data indicate that PS2 is an endogenous inhibitor that dampens the responses of these enzymes to signals.

How do these results relate to the apoptotic actions of wildtype presenilins? As mentioned above, we have previously observed that wildtype PS2 does not directly induce apoptosis in neurons, but rather enhances apoptosis induced by trophic withdrawal or an oxidative challenge when it is overexpressed. This paradigm fits quite nicely if we focus on the dramatic inhibition of NF κ B by PS2. The transcription factor NF κ B does not itself induce apoptosis, rather its expression appears to protect

the cell against apoptosis. Based on these findings, we might expect that inhibition of NF κ B activity by PS2 would enhance apoptosis induced through a parallel pathway, such as apoptosis induced by inhibition of PKB due to trophic withdrawal, but would not induce apoptosis directly.

What remains unclear is why inhibition of JNK by overexpression of PS2 would not exert an inhibitory influence on apoptosis. Perhaps inhibition of JNK only inhibits apoptosis when other pro-apoptotic pathways are not activated. An alternative explanation may lie in experimental details. Previous studies have shown that inhibition of the JNK cascade inhibits apoptosis, however this work was done under conditions where NF κ B activity was not also inhibited (Xia *et al.*, 1995). The behavior of the apoptotic pathways during conditions of combined JNK/NF κ B inhibition by PS2 may account for the ability of PS2 to enhance apoptosis.

Presenilin Mutations: Loss of function exaggerates JNK/NF κ B responses

Having presented the basic biology of presenilins, as it relates to apoptosis, we can now turn to examine the affect of mutations on presenilin function. The presenilins came to the attention of the Alzheimer community because mutations in the presenilins produce an early onset, familial form of Alzheimer disease. Two types of Alzheimer-related mutations have been identified in PS2, M136V and N141I, while multiple Alzheimer-related mutations have been identified in PS1. Rapidly after the identification of the presenilins, Younkin and colleagues noted that cells expressing mutant forms of presenilins produce up to 3 times as much A β ₁₋₄₂, although the amount of A β ₁₋₄₀ was unchanged (Scheuner *et al.*, 1996). Since mutations in presenilins appear to uniformly increase secretion of A β ₁₋₄₂, these results set a standard, which requires that in order for a presenilin function to be considered relevant to the pathogenesis of Alzheimer's disease, all Alzheimer related presenilin mutations must affect that function. Moreover, because of the strong connection between A β and Alzheimer's disease, any function relating presenilins to Alzheimer disease must somehow account for the consistent changes in A β (increased secretion in cell culture and accumulation *in vivo*) seen in the disease. It is currently unclear whether the connection between presenilins and apoptosis will meet this standard and answer mechanistic questions relating to A β and Alzheimer's disease. However, what is clear is that all of the mutations analyzed to date do alter the apoptotic-related functions of presenilins.

Based on the results presented above, it is clear that PS2 regulates signal transduction pathways controlling apoptosis, and it appears likely that PS1 also impacts on these same pathways. Interestingly, where it has been examined, the Alzheimer-associated presenilin mutations all appear to increase the apoptotic activity of the presenilins (Deng *et al.*, 1996; Guo *et al.*, 1997; Wolozin *et al.*, 1996). Wildtype PS2 enhances induced by stressors, but does not appear to induce apoptosis by itself. However, using a transient transfection paradigm, we found that the N141I mutation of PS2 can induce apoptosis without an apoptotic stimulus. Transfection of neuronally differentiated PC12 cells with N141I PS2 is toxic to most of the cells taking up the cDNA. It is possible, though, to generate cell lines that stably express PS2. Using those cell lines we have found that the cells that are able to grow up expressing the mutant N141I PS2 are still more prone to apoptosis. Although they can proliferate, the cells show an increased tendency to die in response to a stressor such as TNF α . Mattson's group has noted a similar pattern of behavior with PS1 constructs (Guo *et al.*, 1996; Guo *et al.*, 1997). Although PC12 cells expressing wildtype PS1 do not show increased apoptosis in response to stressors such as

A β or trophic withdrawal, cells expressing the L286V mutant PS1 show a 2–10 fold increase in the amount of apoptosis in response to trophic withdrawal or A β treatment. The increased sensitivity was present whether the PS1 was stably or transiently expressed, and whether DNA fragmentation or MTT reduction was used as an endpoint. We have observed a similar pattern of behavior in Jurkat cells, a line derived from T-cells, expressing the H115Y PS1 (Figure 6). These cells show approximately a 3-fold increase in sensitivity to ceramide, TNF α or Fas. Interestingly, in the Jurkat cell line, the increased sensitivity is observed using a MTT assay, but not using a DNA fragmentation assay. These results present clear evidence that the mutations in presenilins that are associated with Alzheimer's disease increase the sensitivity of cells to apoptosis. However, before the connection between presenilins, apoptosis and Alzheimer's disease are as clear as they are for A β more types mutations must be examined.

The effects of these mutations on the NF κ B/JNK signal transduction pathway are fascinating. The function of wildtype PS2 is to inhibit the NF κ B/JNK pathway. It turns out that the N141I and M239V mutations in PS2 both result in molecules that are largely inactive (Figure 7). Under basal conditions, cells over-expressing the mutant PS2 cDNAs show little inhibition of NF κ B or JNK, unlike the dramatic inhibition seen with wildtype PS2. This may not be problematic under basal conditions, but the lack of the inhibitory action of PS2 produces dramatic effects following cellular stimulation. Cells that have the 'inactive' mutant PS2 constructs are hyper-reactive. When challenged with a stressor, such as A β (or TNF α or PDBU/ionomycin), cells expressing the A β receptor, RAGE, and a mutant PS2 show a 2.5–8 fold greater response than control cells and a 25–40 fold greater response than cells overexpressing wildtype PS2 (depending on the stressor). In the case of the differentiated PC12 cells that were used to analyze PS2 function, NGF-mediated stimulation of the p75 receptor may become a lethal trigger of the JNK cascade in cells expressing mutant PS2. In the same way that dominant negative JNK constructs can prevent apoptosis, so to could over-stimulation of the JNK cascade induce apoptosis. Thus, the increased sensitivity of cells expressing mutant PS2 may lie in the hyper-responsiveness of the JNK/NF κ B cascades.

In order to fully understand how abnormalities in this system would affect cellular biology, the inter-relationship of the JNK and NF κ B cascades need to be studied in more detail. Blockade of JNK clearly can inhibit apoptosis, and significant over-expression of the NF κ B dimer also reduces apoptosis, but what of hyperstimulation of both cascades, as occurs with the presenilin mutations?

Mutations that result in loss of function typically produce recessive disorders, while mutations that lead to a gain of function produce a genetically dominant disorder. Presenilin-mediated familial Alzheimer's disease is a dominant disorder suggesting a gain of function. But the multiple sites producing familial Alzheimer's disease is more consistent with a loss of function because it is difficult to imagine how so many different points in a protein could increase its function. However, with the discovery that presenilins normally inhibit the action of the JNK and NF κ B cascades, this dichotomy is clarified. The loss of inhibitory presenilin action that is associated with presenilin mutations leads to a gain of activity in the JNK and NF κ B cascades. This system is analogous to the effect of mutation in p53 or the retinoblastoma protein. These proteins function to delay the cell cycle in order to allow DNA repair. However, loss of function of p53 or the retinoblastoma rb protein allows the cell to progress without DNA repair and to proceed into an oncogenic cycle. Thus, loss of function produces a dominant gain of cell cycle proliferative ability and a dominant disorder. Although Alzheimer's

disease is not a proliferative disorder, loss of presenilin function allows increased activity of the JNK and NF κ B cascades, with the concomitant increases in apoptotic sensitivity and increases in transcriptional activity. Thus, loss of presenilin function produces a dominant gain of function.

INTEGRATION WITH ALZHEIMER PATHOPHYSIOLOGY

There is a strong consensus within the community of Alzheimer researchers that A β is central to the pathophysiology of AD. If we take this 'amyloid hypothesis' as a given then AD pathophysiology can be simplified into three categories: factors affecting A β production, factors affecting A β aggregation/accumulation and factors affecting A β toxicity. The observations that presenilin mutations increase neuronal vulnerability provide a strong link between presenilins and A β toxicity. Presenilins, though, clearly also have a strong effect on the production of A β ₁₋₄₂. Perhaps these two seemingly disparate observations are actually linked.

Although the evidence linking apoptosis with AD is not as strong as it is for A β and AD, the amount of evidence is increasing rapidly. Several studies have shown that the number of apoptotic neurons in increased up to 50-fold over that in age-matched control brains (Su *et al.*, 1994). One might wonder why all the neurons in a postmortem brain don't show DNA fragmentation. The reason is probably because the apoptotic machinery takes 24–48 hrs to become activated in the brain. Thus, when a normal person dies, the apoptotic machinery does not have time to become active before the neurons die—excitotoxicity and necrosis are presumably the predominant forms of cell death postmortem. Thus, the observation of increased rates of apoptosis in the Alzheimer brain is providing us with information indicating that Alzheimer-neurons are in an unusual metabolic state—one in which apoptotic pathways have been activated. Evidence for the involvement of apoptotic pathways in AD comes from other directions as well. Interestingly, the 717 mutations in amyloid precursor protein that cause familial AD also render neurons vulnerable to apoptosis—independent of the increases in A β production (Yamatsuji *et al.*, 1996). Thus, multiple lines of evidence suggest that molecular changes that activate cell death pathways, in particular apoptotic pathways, also appear to cause AD.

The skeptic might argue that it is no great surprise that cells die in the Alzheimer brain, so why should we care how they die? The mechanism of cell death is significant because it directs our attention to the particular biochemical pathways that control apoptosis, which provides us with a model for understanding how the neurons in the Alzheimer brain die. As this model becomes more refined, it can be used to design pharmaceutical methods for intervening in the cell death process. At each step in the model, the Alzheimer-related genes identified by molecular genetics help us to choose which specific biochemical pathway, among the myriad of possibilities, is the most significant for the pathophysiology of AD. The biology of the presenilins provides us with such a clue.

Although our knowledge of presenilin biology is only beginning to be understood, the initial indications are that these proteins play an essential role in the regulation of the JNK/ NF κ B cascade. The JNK/NF κ B cascade includes the kinases rac, MEKK and JNK. However, since rac stimulates mitochondrial hydrogen peroxide production which activates NF κ B, hydrogen peroxide should also be considered part of this cascade. Hydrogen peroxide, and the free radicals that derive from it, are typically considered to be nuisance chemicals that cause protein oxidation. However, it is important to realize that these are probably functioning predominantly as second messengers that regulate the

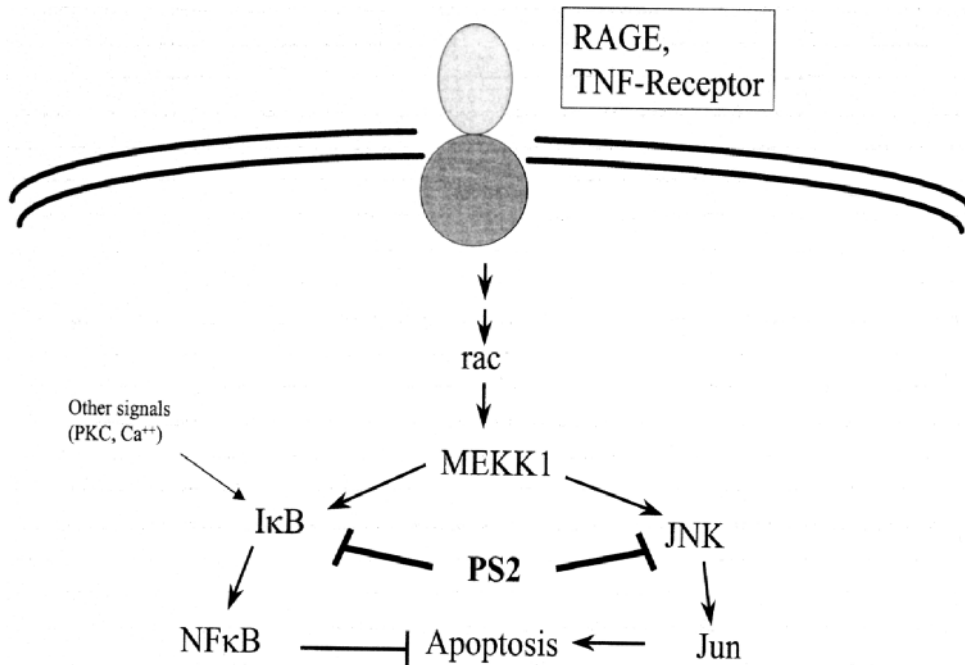


Figure 8. Summary scheme. PS2 appears to act as a shock absorber for the JNK/NFκB apoptotic cascade. Wildtype PS2 inhibits both of these cascade by up to 90%, while PS1 also inhibits, but to a lesser extent. Mutations in PS2 lead to a loss of activity which increases cellular JNK/NFκB responses to stressful stimuli.

activity of multiple enzymes by controlling the state of oxidation. Interestingly, Aβ also stimulates mitochondrial hydrogen peroxide production (Behl *et al.*, 1994). Addition of Aβ to PC12 cells or hippocampal neurons generates hydrogen peroxide that is detectable within 3 hrs with the dye dichlorofluorescein diacetate, which deposits in the mitochondria as it is oxidized. Not surprisingly, NFκB, which is stimulated by hydrogen peroxide, is activated along the same time course following treatment of cells with Aβ (Behl *et al.*, 1994).

Recent work on the genetics of the mitochondrial enzyme, cytochrome C oxidase, have identified an allele that increases the risk of AD, and is present in 50% of the patients with AD (Davis *et al.*, 1997). Cytochrome c oxidase has been previously linked to cell death processes (Papadopoulou and Tsiftoglou, 1996). The polymorphisms in this allele might increase the risk of AD by lowering the threshold for uncoupling and thereby increasing mitochondrial free radical production or by lowering the threshold for release of cytochrome C into the cytoplasm, which is also a trigger for apoptosis (Davis *et al.*, 1997). Free radicals are problematic in two ways. One effect is to induce oxidation. However, a second effect of free radical production is to stimulate the JNK/NFβB cascade and stimulate apoptosis. Thus, it is quite likely that the polymorphisms in the cytochrome C gene affect cell death pathways such as the JNK/NFκB pathway. Together these disparate lines of research are coalescing to paint a picture suggesting that the pathophysiology of AD is intimately connected with

activation of apoptotic pathways (Figure 8). Perturbation of any step along this pathway affects apoptosis. Each step is a potential entry point at which a toxin or a genetic mutation can alter the pathway and render neurons more susceptible to apoptosis. $A\beta$ stimulates the JNK/NF κ B cascade by increasing hydrogen peroxide production. Polymorphisms in cytochrome C oxidase may alter the electron transport chain and lower the threshold for activating the apoptotic pathways. Finally, mutations in presenilins renders the JNK/NF κ B cascade over-reactive. In this sense, AD is the mirror image of cancer. In cancer cell growth pathways are inappropriately activated; at each step of the signal transduction cascade genetic mutations that alter the regulation can induce the cancer. Similarly, in AD, cell death pathways are inappropriately activated; at each step of the signal transduction cascade genetic mutations or toxins that alter the regulation can induce AD.

Integrating Apoptosis with Amyloid

The relationship between apoptosis and production of $A\beta_{1-42}$ is conspicuously omitted from the model presented above, but may become incorporated as our understanding the regulation of $A\beta_{1-42}$ production increases. The simplest explanation for the increases in $A\beta_{1-42}$ associated with the presenilin mutations does not involve apoptosis-associated signal transduction pathways. This model proposes that the mutations reduce the ability of presenilins to bind APP and this leads to altered proteolytic processing. Initial studies suggest that presenilins do indeed directly bind APP, but whether this binding is biologically significant, can regulate APP processing and is altered by the presenilin mutations remains unclear (Weidemann *et al.*, 1997; Xia *et al.*, 1997). Since presenilins are much less abundant than APP it is unclear how the small amount of presenilins might direct trafficking of the larger amount of APP. In addition, the initial studies with presenilins have not detected mutation-related changes in the ability of presenilins to bind APP, although future more detailed studies may yet identify more subtle mutation-related changes in PS/APP binding.

The increased production of $A\beta_{1-42}$ could also be explained by another model, though. In this model the changes in the JNK/NF κ B signal transduction cascade associated with the presenilin mutations alter the processing of APP and increase production $A\beta_{1-42}$. The mechanism remains unclear, but it is relatively simple to imagine that changes in free radical production that are associated with activation of the JNK cascade could lead to changes in $A\beta_{1-42}$ processing. Alternatively, changes transcriptional regulation by NF κ B or c-Jun, could affect the levels of proteases that control APP processing increasing production of $A\beta_{1-42}$. LeBlanc has noted that total $A\beta$ levels increase during apoptosis (LeBlanc, 1995). The presenilin mutations, though, specifically increase production of $A\beta_{1-42}$ (Scheuner *et al.*, 1996). The control of $A\beta_{1-42}$ is beginning to be elucidated. Studies by Younkin and collaborators indicate that exposure of cells to hydrogen peroxide increase the amount of $A\beta_{1-42}$ present in a cell (Younkin, personal communication). This suggests that production of $A\beta_{1-42}$ is regulated by free radicals. Other studies support the idea that free radicals play a role in the regulation of APP biology. Both APP and $A\beta$ normally bind metals, and these metals can serve as catalysts for oxidizing APP or $A\beta$. The presence of this type of control emphasizes the potential importance of free radicals in regulating protein function. Ihara and colleagues have recently shown a mechanism by which changes in calcium metabolism could also affect the production of $A\beta_{1-42}$. They noted that inhibition of calpain, a calciumsensitive protease, increases $A\beta_{1-42}$ production. Thus, an increasing

amount of evidence indicates that production of $A\beta_{1-42}$ is responsive to signal transduction cascades. Since there is strong evidence that the Alzheimer-associated mutations in presenilins alter the regulation of the apoptotic cascades, it appears increasingly likely that these changes could indeed increase the production of $A\beta_{1-42}$.

FUTURE DIRECTIONS

The work presented above indicates that there is a strong connection between presenilins and the apoptotic cascades. We used this information to determine that the presenilins are important regulators of the JNK and NF κ B cascades. It remains to be seen, though, whether the presenilins only regulate the JNK and NF κ B cascades or have broader roles in cellular functioning. The observation that NOTCH signaling is impaired in the presenilin 1 knock-out mouse, suggests that PS1 may regulate more than the JNK/NF κ B cascade. Never-the-less, until we know how the presenilins regulate these cascades we won't know how much potential there is for broader regulation and crosstalk. For instance, do presenilins bind directly to enzymes of the JNK/NF κ B cascade, or do they somehow regulate a second messenger that is involved in JNK/NF κ B activation? The fact that I κ B is cytoplasmic and PS2 is membrane bound suggests that the two proteins may not directly interact, although this remains to be determined. Thus, although we have made significant progress in identifying the physiological connections between the enzymes, we have yet to determine the biochemical partners that directly bind to the presenilins.

The identification of the role of presenilins in regulating apoptosis adds to an increasing number of neurodegenerative diseases that may occur due to abnormal activation of apoptosis. NF κ B appears to be activated in Parkinson's disease (Hunot *et al.*, 1997). Approximately 5% of neurons in the substantia nigra show nuclear NF κ B staining, which is a sign of NF κ B activation. In contrast, only 0.07% of neurons from the substantia nigra in control brains showed nuclear NF κ B (Hunot *et al.*, 1997). Recently, the mutations in the protein synuclein have been shown to cause a familial form of Parkinson's disease that shows an autosomal dominant form of inheritance (Polymeropoulos *et al.*, 1997). It is entirely conceivable that these mutations may somehow lead to activation of the apoptotic cascades in the Parkinson brain. Familial forms of amyotrophic lateral sclerosis results from mutations in superoxide dismutase, the enzyme that regulates superoxide and hydrogen peroxide levels (Deng *et al.*, 1993; Rosen *et al.*, 1993). These mutations lead to increased apoptosis in the lateral motor tracts of the spinal cord (Rabizadeh *et al.*, 1995). Finally, apoptosis is also increased following traumatic injury. Following trauma to the spinal cord, there is an immediate loss of function, but the loss of function actually increases for 1–2 weeks following the injury. Recent studies show that there is a parallel increase in apoptosis in the spinal cord that may account for these changes (Crowe *et al.*, 1997).

As the work with the presenilins shows, though, identifying the role of apoptotic processes in neurodegenerative disorders is only the first step that greatly facilitates identification of the biochemical processes underlying these disorders. In the case of presenilins, identification of their role in apoptosis has pointed towards the JNK/NF κ B pathways. Although it is likely that our knowledge of presenilin biochemistry will continue to evolve, we may be able to use this knowledge to develop novel treatments for AD. Similarly, knowledge of the biology of the apoptotic pathways activated in other neurodegenerative disorders can be used to develop pharmaceutical strategies to these diseases.

Multiple strategies are being tried. One promising strategy is the use of caspase inhibitors. Caspases are the 'executioners' of apoptosis; these are the proteases that appear to be the final target of the apoptotic cascades. Inhibition of caspases, with the membrane permeable peptide inhibitors DVED-CH₂F, YVAD-CH₂F or boc-A-CH₂F has been shown to inhibit apoptosis, *in vitro* (Lynch *et al.*, 1997; Nicholson *et al.*, 1995). Inhibition of caspases *in vivo* doubles the length of survival after the appearance of the illness in transgenic mice expressing the M17Z mutant superoxide dismutase gene, associated with ALS. Transgenic mice expressing this mutant develop the same pathology as patients with ALS and die within 11 days, however, transgenic mice expressing the M17Z superoxide dismutase and a dominant negative ICE construct develop the disease at the same time but live for 27 days. Another approach being used is use of the apoptotic inhibitor, Bcl-2, which can be introduced with viral vectors, to inhibit the apoptotic process. For instance, Sapolsky and colleagues have used Bcl-2 viral vectors to block neuronal cell death induced by the neurotoxin 6-OH dopamine (Lawrence *et al.*, 1996). Although Sapolsky has observed some efficacy, the protective effects are mainly seen if the Bcl-2 viral vector is given prior to the chemical insult. The problem is that there is a window of about 8 hours after application of the virus before the Bcl-2 protein is made during which the cells are vulnerable (Lawrence *et al.*, 1996). However, the field of viral vectors will no doubt continue to evolve. Pharmacotherapy based on inhibition of apoptosis is still in its infancy but may become increasingly important in preventing neurodegenerative diseases.

Summary

In summary, there is strong evidence that both presenilins participate in the signal transduction cascades that regulate apoptosis. Wildtype presenilin 2 participates enhances apoptosis induced by trophic withdrawal, and mutations in both presenilins sensitize cells to cell death induced by a wide range of factors, including A β , trophic withdrawal, tumor necrosis factor and ceramide. The explanation for this profile of biochemical sensitivity is clear upon analysis of the signal transduction pathways regulated by presenilins. These proteins are endogenous inhibitors of the JNK/NF κ B cascade. PS2 strongly inhibits both JNK and NF κ B activity. Loss or gain of activity is problematic for the cells. Overexpression of wildtype PS2 greatly reduces NF κ B activity which removes an important neuroprotective factor from the cell and renders the cell vulnerable to cell death. Mutant forms of PS1 and PS2 are both inactive. This removes an endogenous 'shock absorber' from the cells and renders the cell hyper-responsive to agents that activate the JNK cascade. This hyper-responsiveness renders the cells vulnerable to stress. Thus, mutations in presenilins could directly increase cell death in AD. An important question, which has yet to be answered, is whether the changes in signal transduction cascades induced by presenilins are also responsible for the increases in A β ₁₋₄₂ that may drive AD.

REFERENCES

- Alexander, P., Kohn, A., Palacino, J., Schultz, R., Haas, C., Takashima, A., St. George-Hyslop, P., Hardy, J., Yan, S., Stern, D., and Wolozin, B. (1997) Presenilin 2 is an endogenous Inhibitor of the NF κ B/JNK pathway, (submitted).

- Alkalay, I., Yaron, A., Hatzubai, A., Orian, A., Ciechanover, A., and Ben-Neriah, Y. (1995) Stimulation-dependent I κ B α phosphorylation marks the NF- κ B inhibitor for degradation via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. USA*, **92**, 10599–603.
- Ankarcrona, M., Dypbukt, J.M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S.A., and Nicotera, P. (1995) Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. *Neuron*, **15**, 961–73.
- Asahi, M., Hoshimaru, M., Uemura, Y., Tokime, T., Kojima, M., Ohtsuka, T., Matsuura, N., Aoki, T., Shibahara, K., and Kikuchi, H. (1997) Expression of interleukin-1 beta converting enzyme gene family and bcl-2 gene family in the rat brain following permanent occlusion of the middle cerebral artery. *Journal of Cerebral Blood Flow, and Metabolism*, **17**, 11–8.
- Baldwin, A.J. (1996) The NF κ B and I κ B proteins: new discoveries and insights. *Annual Review of Immunology*, **14**, 649–83.
- Beg, A.A., and Baltimore, D. (1996) An Essential Role For Nf-Kappa-B In Preventing Tnf-Alpha-Induced Cell Death. *Science*, **274**, 782–784.
- Behl, C., Davis, J., Lesley, R., and Schubert, D. (1994) Hydrogen peroxide mediates amyloid β protein toxicity. *Cell*, **77**, 1–20.
- Caelles, C., Helmborg, A., and Karin, M. (1994) p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. *Nature*, **370**, 220–3.
- Chinnaiyan, A., O'Rourke, K., Lane, B., and Dixit, V. (1997) Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. *Science*, **275**, 1081–2.
- Chittenden, T., Harrington, E., O'Connor, R., Flemington, C., Lutz, R., Evan, G., and Guild, B. (1995) Induction of apoptosis by the Bcl-2 homologue Bak. *Nature*, **374**, 733–6.
- Cobb, M., Boulton, T., and Robbins, D. (1991) Extracellular signal-regulated kinases: ERKs in progress. *Cell Reg.*, **2**, 965–78.
- Crespo, P., Xu, N., Simonds, W., and Gutkind, J. (1994) Ras-dependent activation of MAP kinase pathway mediated by G-protein $\beta\gamma$ subunits. *Nature*, **369**, 418–20.
- Crowe, M., Bresnahan, J., Shuman, S., Masters, J., and Beattie, M. (1997) Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. *Nature Med.*, **3**, 73–76.
- Darmon, A., Nicholson, D., and Bleackley, R. (1995) Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature*, **377**, 446–8.
- Darzynkiewicz, Z., Li, X., and Gong, J. (1994) Assays of cell viability: discrimination of cells dying by apoptosis. *Methods in Cell Biol.*, **41**, 15–38.
- Davis, R., Miller, S., Herrnstadt, C., Ghosh, S., Fahy, E., Shinobu, L., Galasko, D., Thal, L., Beal, M., Howell, N., and Parker, W. (1997) Mutations in mitochondrial cytochrome c oxidase genes segregate with late-onset Alzheimer disease. *PNAS*, **94**, 4526–31.
- Deng, G., Pike, C., and Cotman, C. (1996) Alzheimer-associated presenilin-2 confers increased sensitivity to apoptosis in PC12 cells. *FEBS Lett.*, **397**, 50–54.
- Deng, H., Hentati, H., Tainer, J., Cayabyab, A., Hung, W., Getzoff, E., Pericak-Vance, M., and Siddique, T. (1993) Amyotrophic lateral sclerosis and structural defects in Cu, Zn superoxide dismutase. *Science*, **261**, 1047–51.
- Doan, A., Thinakaran, G., Borchelt, D.R., Slunt, H.H., Ratovitsky, T., Podlisny, M., Selkoe, D.J., Seeger, M., Gandy, S.E., Price, D.L., and Sisodia, S.S. (1996) Protein Topology Of Presenilin 1. *Neuron*, **17**, 1023–1030.
- Duan, H., and Dixit, V. (1997) RAIDD is a new 'death' adaptor molecule. *Nature*, **385**, 86–9.

- Dudek, H., Datta, S.R., Franke, T.F., Birnbaum, M.J., Yao, R., Cooper, G.M., Segal, R.A., Kaplan, D.R., and Greenberg, M.E. (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt [see comments]. *Science*, **275**, 661–5.
- Elder, G., Tezapsidis, N., Carter, J., Shioi, J., Bouras, C., Li, H., Johnston, J., Efthimiopoulos, S., Friedrich, V., and Robakis, N. (1996) Identification and neuron specific expression of the S182/ Presenilin I protein in human and rodent brains. *J. Neurosci. Res.*, **45**, 308–20.
- Ellis, H., and Horvitz, H. (1986) Genetic control of programmed cell death in the nematode *Caenorhabditis elegans*. *Cell*, **44**, 817–29.
- Ellis, R., Yuan, J., and Horvitz, H. (1991) Mechanisms and function of cell death. *Ann. Rev. Cell Biol.*, **7**, 663–98.
- Enari, M., Talanian, R., Wong, W., and Nagata, S. (1996) Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. *Nature*, **380**, 723–6.
- Estus, S., Zaks, W.J., Freeman, R.S., Gruda, M., Bravo, R., and Johnson, E.J. (1994) Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. *J. Cell Biol.*
- Franke, T., Yang, S., Chan, T., Datta, K., Kaziauskas, A., Morrison, D., Kaplan, D., and Tsichlis, P. (1995) The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell*, **81**, 727–36.
- Gavrieli, Y., Sherman, Y., and Ben, S.S. (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol.*, **119**, 493–501.
- Glucksmann, A. (1951) Cell death in normal vertebrate ontogeny. *Biol. Rev.*, **26**, 59–86.
- Gold, R., Schmied, M., Giegerich, G., Breitschopf, H., Hartung, H., Toyka, K., and Lassmann, H. (1994) Differentiation between cellular apoptosis and necrosis by combined use of *in situ* tailing and nick translation techniques . *Lab. Invest.*, **2**, 219–25.
- Guo, Q., Furukawa, K., Sopher, B., Pham, D., Xie, J., Robinson, N., Martin, G., and Mattson, M. (1996) Alzheimer PS-1 mutation perturbs calcium homeostasis and sensitizes PC12 cells to death induced by amyloid β -peptide. *NeuroReport*, **8**, 379–83.
- Guo, Q., Sopher, B., Furukawa, K., Pham, D., Robinson, N., Martin, G., and Mattson, M. (1997) Alzheimer's presenilin mutation sensitizes neural cells to apoptosis induced by trophic factor withdrawal and amyloid β -Peptide: Involvement of calcium and oxyradicals. *J. Neurosci.*, **17**, 4212–22.
- Hamburger, V. (1958) Regression versus peripheral control of differentiation in motor hypoplasia. *Am. J. Anat.*, **102**, 365–410.
- Hemmings, B.A. (1997) Akt signaling: linking membrane events to life and death decisions [comment]. *Science*, **275**, 628–30.
- Hengartner, M.O., and Horvitz, H.R. (1994) *C. elegans* cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell*, **76**, 665–76.
- Hockenbery, D.M., Oltvai, Z.N., Yin, X.-M., Millman, C.L., and Korsmeyer, S.J. (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell*, **75**, 241–51.
- Hunot, S., Brugg, B., Ricard, D., Michel, P., Muriel, M., Ruberg, M., Faucheux, B., Agid, Y., and Hirsch, E. (1997) Nuclear translocation of NF- κ B is increased in dopaminergic neurons of patients with Parkinson disease. *PNAS*, **94**, 7531–6.
- Jacobson, M., Weil, M., and Raff, M. (1997) Programmed cell death in animal development. *Cell*, **88**, 347–54.
- Johnson, G., and Vaillancourt, R. (1994) Sequential protein kinase reactions controlling cell growth and differentiation. *Curr. Op. Cell Biol.*, **6**, 230–8.

- Kauffmann-Zeh, A., Rodriguez-Viciana, P., Ulrich, E., Gilbert, C., Coffey, P., Downward, J., and Evan, G. (1997) Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature*, **385**, 544–8.
- Kayalar, C., Ord, T., Testa, M., Zhong, L., and Bredesen, D. (1997) Cleavage of actin by interleukin 1 β -converting enzyme to reverse DNase I inhibition. *PNAS*, **93**, 2234–8.
- Kennedy, S.G., Wagner, A.J., Conzen, S.D., Jordan, J., Bellacosa, A., Tsichlis, P.N., and Hay, N. (1997) The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes, and Development*, **11**, 701–13.
- Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer*, **26**, 239–57.
- Kim, T., Pettingell, W., Hallmark, O., Moir, R., Wasco, W., and Tanzi, R. (1997a) Endoproteolytic cleavage and proteosomal degradation of presenilin 2 in transfected cells. *J. Biol. Chem.*, **272**, 11006–10.
- Kim, T., Pettingell, W., Jung, Y., Kovacs, D., and Tanzi, R. (1997b) Alternative cleavage of Alzheimer-associated presenilins during apoptosis by a caspase-3 family protease. *Science*, **277**, 373–6.
- Kinoshita, M., Tomimoto, H., Kinoshita, A., Kumar, S., and Noda, M. (1997) Up-regulation of the Nedd2 gene encoding an ICE/Ced-3-like cysteine protease in the gerbil brain after transient global ischemia. *Journal of Cerebral Blood Flow, and Metabolism*, **17**, 507–14.
- Gluck, R.M., Bossy-Wetzel, E., Green, D.R., and Newmeyer, D.D. (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis [see comments]. *Science*, **275**, 1132–6.
- Kohn, A.D., Takeuchi, F., and Roth, R.A. (1996) Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. *J. of Biological Chemistry*, **271**, 21920–6.
- Kovacs, D.M., Fausett, H.J., Page, K.J., Kim, T.W., Moir, R.D., Merriam, D.E., Hollister, R.D., Hallmark, O.G., Mancini, R., Felsenstein, K.M., Hyman, B.T., Tanzi, R.E., and Wasco, W. (1996) Alzheimer-associated presenilins 1 and 2: neuronal expression in brain and localization to intracellular membranes in mammalian cells. *Nature Medicine*, **2**, 224–9.
- Lam, M., Dubyak, G., Chen, L., Nunez, G., Miesfeld, R.L., and Distelhorst, C.W. (1994) Evidence that bcl-2 represses apoptosis by regulating endoplasmic reticulum-associated calcium fluxes. *PNAS*, **91**, 6569–73.
- Lawrence, M., Ho, D., Sun, G., Steinberg, G., and Sapolsky, R. (1996) Overexpression of Bcl-2 with Herpes Simplex Virus vectors protects CNS neurons against neurological insults *in vitro* and *in vivo*. *J. Neuroscience*, **16**, 486–96.
- Lazebnik, Y., Kaufmann, S., Desnoyers, S., Poirer, G., and Earnshaw, W. (1994) Cleavage of poly (ADP-ribose) polymerase by a porteinase with properties like ICE. *Nature*, **371**, 346–7.
- LeBlanc, A. (1995) Increased production of 4 kDa amyloid beta peptide in serum deprived human primary neuron cultures: possible involvement of apoptosis. *Journal of Neuroscience*, **15**, 7837–46.
- Lee, F., Hagler, J., Chen, Z., and Maniatis, T. (1997) Activation of the I κ B α kinase complex by MEKK1, a kinase of the JNK pathway. *Cell*, **88**, 213–22.
- Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D.M., Oshima, J., Pettingell, W.H., Yu, C.E., Jondro, P.D., Schmidt, S.D., Wang, K., *et al.* (1995) Candidate gene for the chromosome 1 familial Alzheimer's disease locus [see comments]. *Science*, **269**, 973–7.
- Liu, Z.G., Baskaran, R., Leachou, E.T., Wood, L.D., Chen, Y., Karin, M., and Wang, J.Y.J. (1996) Three Distinct Signalling Responses By Murine Fibroblasts to Genotoxic Stress. *Nature*, **384**, 273–276.
- Lynch, T., Vasilakos, J., Raser, K., Keane, K., and Shivers, B. (1997) Inhibition of the interleukin1 β converting enzyme family rescues neurons from apoptotic death. *Mol. Psych.*, **2**, 227–38.
- Martin, S., Green, D., and Cotter, T. (1994) Dicing with death: dissecting the components of the apoptosis machinery. *TIBS*, **19**, 26–30.

- Mashima, T., Naito, M., Fujita, N., Noguchi, K., and Tsuro, T. (1995) Identification of actin as a substrate of ICE and an ICE-like protease and involvement of an ICE-like protease but not ICE in VP-16-induced U937 apoptosis. *Biochem. Biophys. Res. Com.*, **217**, 1185–92.
- Miller, D.K. (1997) The role of the Caspase family of cysteine proteases in apoptosis. *Seminars in Immunology*, **9**, 35–49.
- Nagata, S. (1997) Apoptosis by death factor. *Cell*, **88**, 355–65.
- Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A., *et al.* (1995) Identification and inhibition of the ICE/ CED-3 protease necessary for mammalian apoptosis [see comments]. *Nature*, **376**, 37–43.
- Oltvai, Z.N., Millman, C.L., and Korsmeyer, S.J. (1993) Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, **74**, 609–19.
- Papadopoulou, L.C., and Tsiftoglou, A.S. (1996) Effects of hemin on apoptosis, suppression of cytochrome c oxidase gene expression, and bone-marrow toxicity induced by doxorubicin (adriamycin). *Biochemical Pharmacology*, **52**, 713–22.
- Polymeropoulos, M., Higgins, J., Golbe, L., Johnson, W., Ide, S., Di Iorio, G., Sanges, G., Stenroos, E., Pho, L., Schaffer, A., Lazzarini, A., Nussbaum, R., and Duvoisin, R. (1997) Mapping of a gene for Parkinson's disease to chromosome 4q21-q23. *Science*, 1197–9.
- Rabizadeh, S., Gralla, E.B., Borchelt, D.R., Gwinn, R., Valentine, J.S., Sisodia, S., Wong, P., Lee, M., Hahn, H., and Bredesen, D.E. (1995) Mutations associated with amyotrophic lateral sclerosis convert superoxide dismutase from an antiapoptotic gene to a proapoptotic gene: studies in yeast and neural cells. *Proceedings of the National Academy of Sciences of the United States of America*, **92**, 3024–8.
- Rosen, D., Siddique, T., Patterson, D., Figlewicz, D., Sapp, P., Hentati, A., Rouleau, G., Gusella, J., Horvitz, H., and Brown, R. (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature*, **362**, 59–62.
- Rudel, T., and Bokoch, G.M. (1997) Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. *Science*, **276**, 1571–4.
- Sakhi, S., Bruce, A., Sun, N., Tocco, G., Baudry, M., and Schreiber, S. (1994) p53 induction is associated with neuronal damage in the central nervous system. *Proc. Natl. Acad. Sci. USA*, **91**, 7525–9.
- Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T.D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levylahad, E., Viitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wasco, W., Lannfelt, L., Selkoe, D., and Younkin, S. (1996) Secreted Amyloid Beta-Protein Similar to That In the Senile Plaques Of Alzheimers Disease Is Increased *In vivo* By the Presenilin 1 and 2 and App Mutations Linked to Familial Alzheimers Disease. *Nature Medicine*, **2**, 864–870.
- Schwartz, L., and Milligan, C. (1996) Cold thoughts of death: the role of ICE proteases in neuronal cell death. *TINS*, **19**, 555–63.
- Sen, R., and Baltimore, D. (1986) Inducibility of κ immunoglobulin enhancer-binding protein NF κ B by a posttranslational mechanism. *Cell*, **47**, 921–8.
- Shearman, M., Ragan, C., and Iversen, L. (1994) Inhibition of PC12 cell redox activity is a specific, early indicator of β -amyloid-mediated cell death. *Proc. Natl. Acad. Sci. USA*, **91**, 1470–4.
- Sherrington, R., Rogaev, E., Liang, Y., Rogaeva, E., Levesque, G., Ideda, M., Chi, H., Lin, C., Li, G., Holman, K., Tsuda, T., Mar, L., Foncin, J., Bruni, A., Montesi, M., Sorbi, S., Rainero, L., Pinessi, L., Nee, L., Chumakov, I., Pollen, D., Brookes, A., Sanseau, P., Polinsky, R., Wasco, W., Da Silva, H., Haines, J., Pericak-Vance, M., Tanzi,

- R., Roses, A., Fraser, P., Rommens, J., and St George-Hyslop, P. (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature*, **375**, 754–60.
- Shimizu, S., Eguchi, Y., Kosaka, H., Kamiike, W., Matsuda, H., and Tsujimoto, Y. (1995) Prevention of hypoxia-induced cell death by Bcl-2 and Bcl-xL. *Nature*, **374**, 811–3.
- Su, J., Deng, G., and Cotman, C. (1997) Bax protein expression is increased in Alzheimer's brain: Correlation with DNA damage, Bcl-2 expression, and brain pathology. *J. Neuropath. Exp. Neurology*, **56**, 86–93.
- Su, J.H., Andersen, A.J., Cummings, B.J., and Cotman, C.W. (1994) Immunohistochemical evidence for apoptosis in Alzheimer's disease. *NeuroReport*, **5**, 2529–33.
- Takashima, A., Noguchi, K., Michel, G., Mercken, M., Hoshi, M., Ishiguro, K., and Imahori, K. (1996) Exposure of rat hippocampal neurons to amyloid beta peptide (25–35) induces the inactivation of phosphatidylinositol-3 kinase and the activation of tau protein kinase I/glycogen synthase kinase-3 beta. *Neuroscience Letters*, **203**, 33–6.
- Tewari, M., and Dixit, V.M. (1996) Recent advances in tumor necrosis factor and CD40 signaling. *Current Opinion in Genetics, and Development*, **6**, 39–44.
- Thinakaran, G., Borchelt, D., Lee, M., Slunt, H., Spitzer, L., Kim, G., Ratovitsky, T., Davenport, F., Nordstedt, C., Seeger, M., Hardy, J., Levey, A., Gandy, S., Jenkins, N., Copeland, N., Price, D., and Sisodia, S. (1996) Endoproteolysis of presenilin 1 and accumulation of processed derivatives *in vivo*. *Neuron*, **17**, 181–90.
- Thompson, C. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science*, **267**, 1456–62.
- Van Antwerp, D., Martin, S., Kafri, T., Green, D., and Verma, IM. (1996) Suppression of TNF α -induced apoptosis by NF- κ B. *Science*, **274**, 787–9.
- Verheij, M., Bose, R., Lin, X.H., Yao, B., Jarvis, W.D., Grant, S., Birrer, M.J., Szabo, E., Zon, L.L., Kyriakis, J.M., Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, R.N. (1996) Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. *Nature*, **380**, 75–9.
- Verma, I., Stevenson, J., Schwartz, E., Van Antwerp, D., and Miyamoto, S. (1995) Rel/NF- κ B/I κ B family: intimate tales of association and dissociation. *Genes Devel*, **9**, 2723–35.
- Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C. (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *Journal of Immunological Methods*, **184**, 39–51.
- Vito, P., Lacana, E., and D'Adamio, L. (1996) Interfering with apoptosis: Ca²⁺ binding protein Alg2 and Alzheimer's disease gene Alg-3. *Science*, **271**, 521–5.
- Vito, P., Wolozin, B., Ganjei, K., Iwasaki, K., Lacana, E., and D'Adamio, L. (1996) Requirement of the familial Alzheimer's disease gene PS2 for apoptosis: opposing effect of Alg-3. *J. Biol Chem.*, **271**, 31025–8.
- Wang, C., Mayo, M., and Baldwin, A.J. (1996) TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF- κ B. *Science*, **274**, 784–7.
- Weidemann, A., Paliga, K., Durrwang, U., Czech, C., Evin, G., Masters, C.L., and Beyreuther, K. (1997) Formation of stable complexes between two Alzheimer's disease gene products: presenilin2 and beta-amyloid precursor protein. *Nature Medicine*, **3**, 328–32.
- Wolozin, B., Iwasaki, K., Vito, P., Ganjei, K., Lacana, E., Sunderland, T., Zhao, B., Kusiak, J., Wasco, W., and D'Adamio, L. (1996) Participation of presenilin-2 in apoptosis: Enhanced basal activity conferred by Alzheimer mutation. *Science*, **274**, 1710–3.
- Wood, K., Dipasquale, B., and Youle, R. (1993) *In situ* labeling of granule cells for apoptosis-associated DNA fragmentation reveals different mechanisms of cell loss in developing cerebellum. *Neuron*, **11**, 621–32.

- Wu, D., Wallen, H., and Nunez, G. (1997) Interaction and regulation of subcellular localization of CED-4 by CED-9. *Science*, **275**, 1126–9.
- Wyllie, A., and Kerr, J. (1980) cell death: the significance of apoptosis. *Int. Rev. Cytol.*, **68**, 251–306.
- Xia, W., Perez, R., Koo, E., and Selkoe, D. (1997) Interaction between amyloid precursor protein and presenilins in mammalian cells: Implications for the pathogenesis of Alzheimer disease. *PNAS*, **94**, 8208–13.
- Xia, Z., Dickens, M., Raingeaud, J., Davis, R., and Greenberg, M. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science*, **270**, 1326–31.
- Yamatsuji, T., Matsui, T., Okamoto, T., Komatsuzaki, K., Takeda, S., Fukumoto, H., Iwatsubo, T., Suzuki, N., Asami-Odaka, A., Ireland, S., Kinane, T.B., Giambarella, U., and Nishimoto, I. (1996) G protein-mediated neuronal DNA fragmentation induced by familial Alzheimer's disease-associated mutants of APP. *Science*, **272**, 1349–52.
- Yang, E., Zha, J., Jockel, J., Boise, L.H., Thompson, C.B., and Korsmeyer, S.J. (1995) Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell*, **80**, 285–91.

15.

THE PHOSPHORYLATION OF PRESENILIN PROTEINS

JOCHEN WALTER and CHRISTIAN HAASS

*Central Institute of Mental Health, Department of Molecular Biology, J5, 68159
Mannheim, Germany*

Phosphorylation of proteins is a major biological mechanism for regulating cellular function. Protein phosphorylation is involved in proliferation, growth and differentiation of all eukaryotic cells (for review see Krebs, 1993; Hunter, 1995). The various phosphorylation events are catalyzed by a large number of protein kinases, which phosphorylate their respective protein substrates at specific sites. The action of protein kinases can be reversed by protein phosphatases, which dephosphorylate phosphorylated proteins (for review see Cohen, 1992; Wera and Hemmings, 1995). The phosphorylation state of a protein is therefore determined by the counterplay of both, protein kinases and protein phosphatases, and phosphorylation/dephosphorylation is a powerful mechanism in the transduction and amplification of cellular signals.

Several proteins playing a major role in Alzheimer disease (AD) are also affected by protein phosphorylation/dephosphorylation. It has been shown that activation of protein kinase C (PKC) or protein kinase A (PKA) increase the secretion of soluble β -amyloid precursor protein (β APP), and decreases the generation of the β -amyloid peptide (Buxbaum *et al.*, 1993; Hung *et al.*, 1993). In addition, β APP itself is phosphorylated within its ectodomain and is secreted as a phosphorylated protein (Knops *et al.*, 1993; Hung and Selkoe, 1994; Walter *et al.*, 1997a). Furthermore, β APP can be also phosphorylated within the cytoplasmic domain (Suzuki *et al.*, 1994; Oishi *et al.*, 1997).

The microtubule-associated protein tau, the major constituent of neurofibrillary tangles, which is one of the pathogenic hallmarks of Alzheimer's disease can undergo phosphorylation/dephosphorylation (for review see Goedert, 1993; Trojanowski and Lee, 1995). Interestingly, in neurofibrillary tangles, tau occurs in an aberrantly hyperphosphorylated form (Grundke-Igbal *et al.*, 1986; Biernat *et al.*, 1992; Goedert *et al.*, 1992; see also the article by Brand and Eichenmüller). However, the role of phosphorylation of both proteins, β APP and tau, in the pathogenesis of AD is not yet clarified.

Recently, phosphorylation of the presenilins (PS), proteins involved in familial AD has been described (De Strooper *et al.*, 1997; Seeger *et al.*, 1997; Walter *et al.*, 1996; Walter *et al.*, 1997b; Walter *et al.*, 1998). Here, we will review the differential phosphorylation of the two homologous PS-1 and PS-2 proteins.

PHOSPHORYLATION OF FULL-LENGTH PS PROTEINS

Studies analyzing the phosphorylation of PS proteins overexpressed in cultured cells revealed that

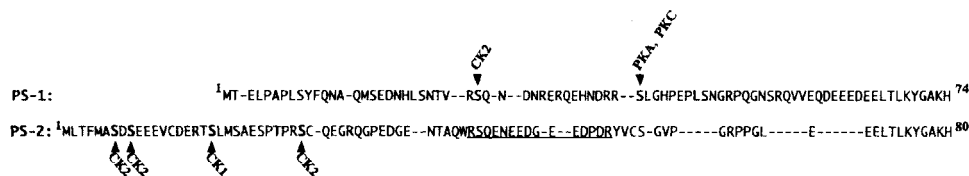


Figure 1. Amino acid sequence of the N-terminal domains of PS-1 and PS-2. Putative recognition sites (Pearson and Kemp, 1991), for PKA (K/R-X-S'/T'), PKC (K/R-X-S'/T' or S'/T'-X-K/R) and protein kinases CK-1 (D/E-X-X-S'/T') and CK-2 (S'/T'-X-X-D/E) are marked by arrowheads (S'/T' are phosphorylatable serine/threonine residues, X stands for any amino acid). The PEST motif in PS-2 is underlined. Serine residues 7, 9 and 19 are *in vivo* phosphorylation sites of PS-2 (Walter *et al.*, 1996).

the homologous PS proteins are differentially phosphorylated *in vivo*. Fulllength PS-2 occurs as a constitutive phosphorylated molecule, and is exclusively phosphorylated on serine residues (Walter *et al.*, 1996; DeStrooper *et al.*, 1997). In contrast, very little if any (Walter *et al.*, 1996) or a variable phosphorylation (DeStrooper *et al.*, 1997) was observed for PS-1. The FAD mutations tested, the A246E mutation of PS-1 and the N141I mutation (volga german) of PS-2, apparently have no effect on the differential phosphorylation of PS-1 and PS-2 (Walter *et al.*, 1996). Since both PS proteins appeared to reside predominantly within the endoplasmic reticulum (Kovacs *et al.*, 1996; Cook *et al.*, 1996; Walter *et al.*, 1996; DeStrooper *et al.*, 1997), differential phosphorylation is not due to distinct subcellular localizations of these proteins. Rather, the differential phosphorylation seems to be determined by structural differences of PS-1 and PS-2. It was found that full-length PS-2 was phosphorylated within its N-terminal domain preceding the first transmembrane region (Walter *et al.*, 1996). Although both PS proteins are highly homologous (Rogeev *et al.*, 1995; Levy-Lahad *et al.*, 1995), their N-terminal domains differ in the primary structure. PS-2 contains a stretch of acidic residues (amino acids 1–20), which is lacking in PS-1 (Figure 1). A search for potential recognition sites of protein kinases revealed that the acidic region in PS-2 contains three consensus sites for casein kinases (CK), one site for CK-1 (serine 19) and two for CK-2 (serines 7 and 9; Figure 1). By mutagenizing these sites to alanine residues, it was demonstrated that all three serine residues (serines 7, 9 and 19) are phosphorylated *in vivo* (Walter *et al.*, 1996). Moreover, *in vitro* phosphorylation demonstrated that the N-terminal domain of PS-2 can be phosphorylated by both kinases, CK-1 and CK-2 (Walter *et al.*, 1996). Thus, it is likely that full-length PS-2 is phosphorylated by CK-1 and CK-2 *in vivo* within its N-terminal domain. The phosphorylated residues within the acidic region of PS-2 precedes a PEST motif (Li and Greenwald., 1996; Rechsteiner and Rogers, 1996), which is lacking in PS-1. PEST sequences have been shown to be implicated in the regulation of protein turnover, e.g. proteins containing a PEST motif undergo enhanced degradation (Rechsteiner and Rogers, 1996). Notably, the activity of PEST sequences can be modulated by phosphorylation (Lin *et al.*, 1996; MacKichan *et al.*, 1996). It will now be of great interest to test whether the phosphorylation of PS-2 influences its turnover.

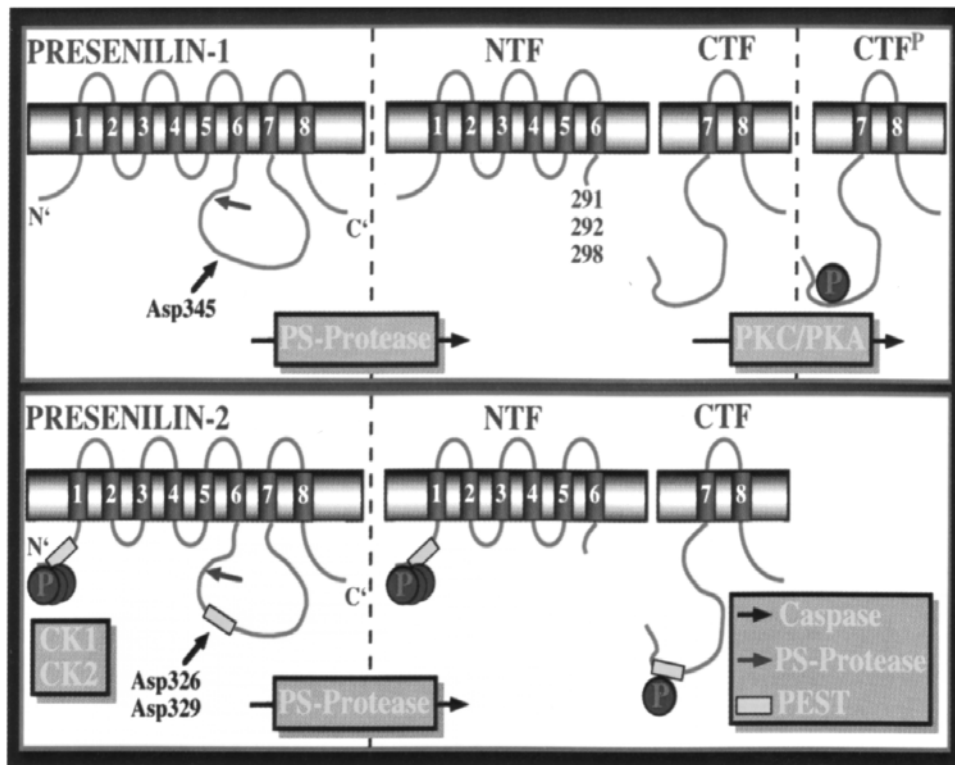


Figure 2. Schematic showing proteolytic processing and phosphorylation of PS-1 and PS-2. PS-1 and PS-2 can be cleaved within the large hydrophilic loop domain between transmembrane domain (TM) 6 and TM7 (according to an eight TM model suggested by Doan *et al.*, 1996), resulting in the generation of NTF and CTF. Cleavage sites for caspases (Asp 345 in PS-1 and Asp 326, 329 in PS-2) are marked by black arrows. The cleavage sites for conventional proteolytic processing are indicated by blue arrows. Phosphorylated domains are marked by blue circles (P). PEST sequences within the N-terminal domain and within the large loop domain of PS-2 are indicated by yellow boxes. Note that full-length PS-1 is not phosphorylated, while full-length PS-2 can be phosphorylated by casein kinases (CK). After conventional cleavage of PS-1, the CTF can be phosphorylated by PKA and/or PKC, while the NTF is not phosphorylated. In contrast to PS-1, the NTF and CTF of PS-2 can be phosphorylated by protein kinases distinct to PKA and PKC (Walter *et al.*, 1998). (See Colour Plate VI)

PHOSPHORYLATION OF PROTEOLYTIC FRAGMENTS OF PS-1

Both PS proteins are cleaved by unknown protease(s) resulting in the generation of heterogeneous ≈ 30 kDa N-terminal and ≈ 20 kDa C-terminal fragments (NTF and CTF, respectively; Mercken *et al.*, 1996; Thinakaran *et al.*, 1996; Kim *et al.*, 1997a; Tomita *et al.*, 1997; see also Figure 2). The cleavage sites in PS-1 were mapped by radiosequencing to amino acids 291, 292, and 298 within an amino acid sequence encoded by exon 10 within the large hydrophilic loop domain (Podlinsky *et al.*, 1997). Interestingly, the NTFs and CTFs are the predominant species of PS proteins detected *in vivo*, while the levels of full-length proteins are apparently very low (Thinakaran *et al.*, 1996). As shown in transgenic mice, FAD-linked mutations in PS-1 result in the hyperaccumulation of the respective

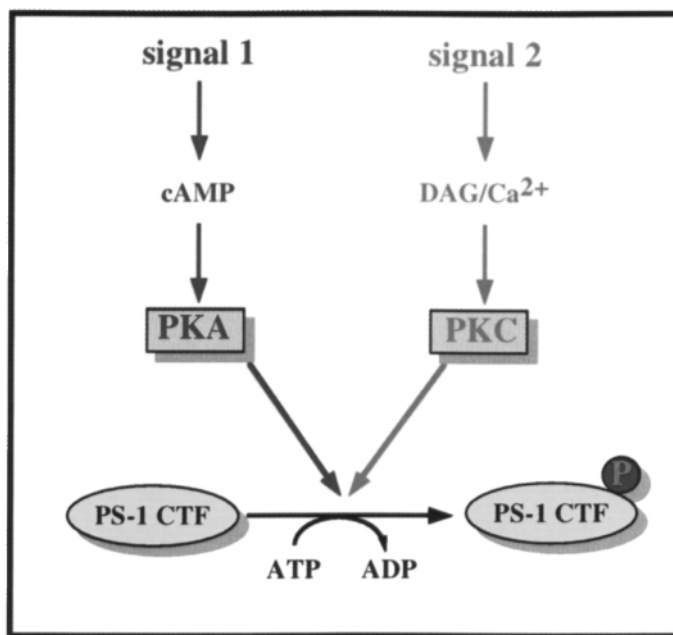


Figure 3. Schematic showing distinct signaling pathways leading to phosphorylation of the PS1 CTF. Phosphorylation can be induced by signal (signal 1) leading to elevated intracellular levels of cAMP, which activates PKA. A distinct pathway can be activated by signals (signal 2) leading to release of PKC activating second messengers (e.g. diacylglycerol (DAG), Ca^{2+}), which also results in phosphorylation of the PS-1 CTF. (See Colour Plate VII)

proteolytic fragments (Lee *et al.*, 1997; see also article by Thinakaran and Sisodia). This might suggest, that the fragments are of a (as yet unknown) physiological and/or pathological function.

Analysis of the proteolytic processing products of PS-1 revealed that the 20 kDa CTF can be phosphorylated *in vivo* (Seeger *et al.*, 1997; Walter *et al.*, 1997b). Phosphorylation of the CTF increases about 4–5 fold upon activation of PKC with phorbol ester. Similar results were obtained upon stimulation of PKA with forskolin which elevates intracellular levels of cAMP. Both, phorbol ester- and forskolin-induced phosphorylation of the PS-1 CTF results in a decreased electrophoretic mobility of the CTF in SDS gels. Phorbol ester induced phosphorylation of the PS-1 CTF can be selectively inhibited by a PKC inhibitor (GF109203X; Toullec *et al.*, 1991), while an inhibitor of PKA (H-89; Chijiwa *et al.*, 1990) had no effect. In contrast, the protein kinase A inhibitor H-89 decreased the forskolin induced phosphorylation, while the PKC inhibitor does not alter the forskolin induced phosphorylation (Walter and Haass, unpublished data). This indicates that phosphorylation of the PS-1 CTF can be mediated by two different signaling pathways *in vivo* (Figure 3). One involves PKA in response to elevated intracellular cAMP levels, and the second involves PKC which can be activated by phorbol ester (Figure 2). Phosphorylation of the PS-1 CTF can also be stimulated by activation of muscarinic acetylcholine receptors (m1 and m3 type receptors) with the muscarinic agonist carbachol (Walter *et al.*, 1997b). Carbachol induced phosphorylation of the PS-1 CTF is selectively suppressed

by GF109203X (Walter *et al.*, 1997b). Therefore, receptor signaling leading to phosphorylation of the PS1 CTF is mediated via PKC.

In contrast to the 20 kDa CTF, the 30 kDa NTF of PS-1 is not phosphorylated. Neither activation of PKC by phorbol ester, nor of PKA by forskolin results in a phosphorylation of the 30 kDa NTF, showing that the proteolytic processing products of PS-1 were differentially modified by phosphorylation (Seeger *et al.*, 1997; Walter *et al.*, 1997b).

Interestingly, the PS-1 holoprotein is not phosphorylated by PKC or PKA, suggesting that phosphorylation of PS-1 can only occur after proteolytic processing. The FAD-linked PS-1 splicing variant, lacking exon 10 (Perez-Tur *et al.*, 1995), which does not undergo proteolytic processing into 20 kDa CTF and 30 kDa NTF (Thinakaran *et al.*, 1996), is also not phosphorylated by PKC or PKA (Walter *et al.*, 1997b). Therefore, normal proteolytic processing is a prerequisite for PKC and PKA mediated phosphorylation of the PS-1 CTF (Figure 2). This might indicate that the proteolytic cleavage results in structural changes of the large loop, allowing phosphorylation of amino acids which are not accessible in the holoprotein.

However, beside the inducible phosphorylation of the PS-1 CTF by phorbol ester or by forskolin, a phosphorylated CTF is also detected in unstimulated cells *in vivo* (Seeger *et al.*, 1997; Walter *et al.*, 1997b). The constitutive phosphorylated CTF has an electrophoretic mobility distinct from that observed for the phosphorylated CTF after PKC or PKA stimulation, suggesting that the CTF of PS-1 can occur in several phosphorylation states. Since the phosphorylation of the PS-1 CTF observed in unstimulated cells is not decreased by selective inhibitors of PKC and PKA (Walter and Haass, unpublished observation), additional protein kinases might be involved in the phosphorylation of the PS-1 CTF.

Several protein kinases were tested to phosphorylate PS-1 *in vitro*. Consistent with the results obtained by *in vivo* phosphorylation, PKA as well as PKC were shown to phosphorylate the hydrophilic loop domain of PS-1. In addition to these second messenger dependent protein kinases, the second messenger independent protein kinases CK-1 and CK-2 also phosphorylate the loop domain of PS-1 *in vitro* (Walter and Haass, unpublished data). Amino acid sequence analysis revealed that the loop domain of PS-1 indeed bears potential recognition sites for CK-1 and CK-2, as well as for PKA and PKC (Figure 4). However, it remains to be determined whether each of these kinases phosphorylates PS-1 *in vivo*.

ALTERNATIVE PROTEOLYTIC PROCESSING IMPAIRS PHOSPHORYLATION OF THE PS-1 FRAGMENTS

In addition to the conventional cleavage of PS proteins, an alternative proteolytic processing pathway has been identified (Kim *et al.*, 1997b; Loetscher *et al.*, 1997; Capell *et al.*, 1997; Hartmann *et al.*, 1997; Grünberg *et al.*, 1998). Alternative cleavage can occur C-terminal to the conventional cleavage sites within exon 11 between aspartate 345 and serine 346 in PS-1 and between aspartate 329 and serine 330 or aspartate 326 and serine 327 in PS-2, respectively. These cleavages increase upon induction of apoptosis and involve cysteine-proteases of the caspase family (Kim *et al.*, 1997b; Grünberg *et al.*, 1998; a detailed description is given in the article by Wasco and Tanzi in this issue).

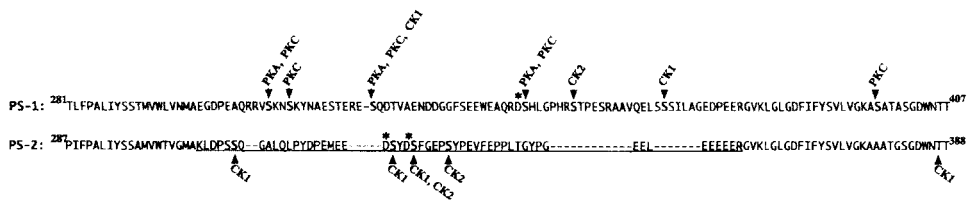


Figure 4. Amino acid sequences of PS-1 and PS-2 loop domains. Putative recognition sites (Pearson and Kemp, 1991) for PKA (K/R-X-S'/T'), PKC (K/R-X-S'/T' or S'/T'-X-K/R) and protein kinases CK-1 (D/E-X-X-S'/T') and CK-2 (S'/T'-X-X-D/E) are marked by arrowheads. Caspase cleavage sites are indicated by *. The amino acid sequence within the PS-2 loop domain comprising a PEST motif is underlined. Note that the PS-2 loop domain does not contain recognition sites for PKA and PKC.

In vivo labeling experiments with ^{32}P -orthophosphate demonstrated that the alternative 14 kDa CTF of PS-1, in contrast to the normal 20 kDa CTF, is not phosphorylated. Neither activation of PKC nor of PKA led to a phosphorylation of the alternative fragment (Walter and Haass, unpublished observation). Therefore, alternative proteolytic processing prevents normal phosphorylation of the CTF. Interestingly, the elongated form of the PS-1 NTF (34 kDa) is phosphorylated *in vivo*, in contrast to the normal 30 kDa NTF. However, phosphorylation of the 34 kDa NTF is independent of PKC and PKA activities as demonstrated by stimulation with phorbol ester or forskolin and by inhibition of both kinases with GF109203X (for PKC inhibition) and H-89 (for PKA inhibition). Since neither the elongated 34 kDa NTF nor the shorter 14 kDa CTF is phosphorylated by PKC or PKA, it is evident that alternative proteolytic processing of PS-1 prevents PKC and PKA regulated phosphorylation of PS-1 fragments. Although the reason for this remains to be determined, there are several possible mechanisms which might explain this phenomenon. First, the subcellular localization of the truncated CTF might be distinct to that of the normal CTF, and the respective protein kinases activities are restricted to the subcellular localization of the normal 20 kDa CTF. Second, the alternative cleavage induces a different conformation of the CTF rendering recognition of the phosphorylation site(s) by the respective protein kinases. Third, cleavage might occur at the protein kinase recognition site, destroying the consensus motif in the primary amino acid sequence. Indeed, one potential phosphorylation site for both, PKC and PKA, is serine 346 which is located directly C-terminal to the alternative cleavage site after aspartate 345 (Figure 4). Serine 346 is preceded by an arginine in-2 position. Cleavage after aspartate 345 would then impair recognition of serine 346 by PKC and PKA preventing its phosphorylation. However, it has to be determined whether serine 345 indeed represents an *in vivo* phosphorylation site.

The functional implications of this complex phosphorylation mechanisms are unclear. Both, PKC and PKA play important roles in cellular function, including the regulation of cell proliferation, differentiation, energy metabolism and protein sorting (for review see Asaoka *et al.*, 1992; Walsh and van Patten, 1994). Notably, proteolytic processing of βAPP was demonstrated to be regulated by protein phosphorylation and is dependent of both, PKA and PKC activities (Buxbaum *et al.*, 1990). Secretion of soluble βAPP increases upon stimulation of PKA or PKC, and in turn generation of β -amyloid peptide ($\text{A}\beta$) decreases (Buxbaum *et al.*, 1993; Hung *et al.*, 1993). Whether PKA and PKC

mediated phosphorylation of the PS-1 CTF is directly involved in regulation of β APP processing will be of great interest.

It was shown that the alternative proteolytic processing route of PS-1 increases upon induction of apoptosis leading to accumulation of the shorter CTF, and PS-1 was characterized as a death substrate for proteases of the caspase family (Grünberg *et al.*, 1998). Interestingly, inhibition of dephosphorylation by okadaic acid results in decreased generation of the shorter PS-1 CTF during apoptosis (Walter and Haass, unpublished observation), indicating that protein phosphorylation regulates alternative cleavage of PS-1.

As demonstrated in primary rat hippocampal neurons and human brain tissue an alternative proteolytic pathway increases during neuronal differentiation, while the generation of 20 kDa CTF and 30 kDa NTF by conventional proteolytic cleavage is decreased (Busciglio *et al.*, 1997; Capell *et al.*, 1997; Hartmann *et al.*, 1997). The complex phosphorylation of PS-1 might represent a highly controlled mechanism to regulate PS processing and function during neuronal development.

PHOSPHORYLATION OF PROTEOLYTIC FRAGMENTS OF PS-2

As described above full-length PS-2 occurs as a constitutively phosphorylated protein. Like the PS-1 holoprotein, PS-2 also undergoes conventional proteolytic processing (Kim *et al.*, 1997a; Tomita *et al.*, 1997; see also Figure 2). In addition, PS-2 can also be cleaved by caspases in an alternative pathway (Kim *et al.*, 1997b; Loetscher *et al.*, 1997). Analysis of the phosphorylation status of the proteolytic processing products of PS-2 revealed that the conventional NTF and CTF occur as phosphorylated polypeptides *in vivo*. In contrast to the PS-1 CTF, phosphorylation of the PS-2 CTF is not mediated by PKC or PKA (Walter *et al.*, 1998; see Figure 2).

The amino acid sequence of the PS-2 loop domain contains a stretch of acidic residues including a cluster of potential phosphorylation sites for protein kinases CK-1 and CK2 (Figure 4). *In vitro* phosphorylation assays using the recombinant loop domain of PS-2 demonstrated that CK-1 and CK-2 can readily phosphorylate this domain. In contrast, PKC and PKA are not effective in phosphorylation of the PS-2 loop which is consistent with the data from *in vivo* phosphorylation, demonstrating that activation of the respective kinases do not increase phosphorylation of the PS-2 CTF. Stoichiometric analysis of the *in vitro* PS-2 loop phosphorylation revealed three phosphorylation sites within that domain. While CK-1 can phosphorylate two distinct sites, CK-2 phosphorylates a single site within the PS-2 loop domain (Walter *et al.*, 1998).

PS-2 has been shown to be implicated in apoptosis. Full-length PS-2 facilitates apoptosis in PC-12 cells and T-lymphocytes (Deng *et al.*, 1996; Vito *et al.*, 1996a; Wolozin *et al.*, 1996). In contrast, a truncated, artificial C-terminal fragment of PS-2 has a protective effect in T-cell-receptor induced apoptosis (Vito *et al.*, 1996b; Wolozin *et al.*, 1996). The alternative proteolytic processing pathway of PS-2, like that of PS-1, is increased during apoptosis (Kim *et al.*, 1997b; Loetscher *et al.*, 1997). It was demonstrated that a FAD causing mutation of PS-2 increases the amount of the alternative CTF. Since levels of A β of 42 amino acid length also is increased in FAD causing mutations, levels of alternative PS-2 CTF and production of the longer 42 amino acid form of A β correlate (Kim *et al.*, 1997b). It is now of great importance to investigate whether alternative processing of PS proteins plays a role in the pathogenesis of AD.

Interestingly, potential phosphorylation sites for CK-1 and CK-2 (serines 327 and 330) are located adjacent to known caspase cleavage sites of PS-2 after aspartates 326 and 329 (Loetscher *et al.*, 1997; Kim *et al.*, 1997b; see also Figure 4). This is in analogy to PS1, in which a potential phosphorylation site for PKC and PKA is located at the cleavage site for caspases as well. It will therefore be of great interest to prove, whether alternative proteolytic processing of PS-2 is also regulated by phosphorylation.

This article aimed to outline the complex modification of PS proteins by phosphorylation. Although both PS proteins are highly homologous in their primary amino acid sequence, it turned out that they are differentially modified by phosphorylation. Both, the full-length proteins and the respective proteolytic processing products are regulated by different mechanisms involving distinct protein kinases. The differential phosphorylation of PS-1 and PS-2 might reflect either different biological functions or distinct regulatory principles of similar properties of these proteins.

REFERENCES

- Asaoka, Y., Nakamura, S., Yoshida, K., and Nishizuka, Y. (1992) Protein kinase C, calcium and phospholipid degradation. *Trends. Biochem. Sci.*, **17**, 414–417.
- Biernat, J., Mandelkow, E.-M., Schröter, C., Lichtenberg-Kraag, B., Steiner, B., Berkling, B., Meyer, H., Mercken, M., Vandermeeren, A., Goedert, M., and Mandelkow, E. (1992) The switch of tau protein to an Alzheimer-like state includes the phosphorylation of two serine-proline motifs upstream of the microtubule binding region. *EMBO J.*, **11**, 1593–1597.
- Busciglio, J., Hartmann, H., Lorenzo, A., Wong, C., Baumann, K., Sommer, B., Staufenbiel, M., and Yankner, B.A. (1997) Neuronal localization of presenilin-1 and association with amyloid plaques and neurofibrillary tangles in Alzheimer disease. *J. Neurosci.*, **17**, 5101–5107.
- Buxbaum, J.D., Gandy, S.E., Cicchetti, P., Ehrlich, M.E., Czernik, A.J., Fracasso, P.R., Ramabhadran, T.V., Unterbeck, A.J., and Greengard, P. (1990) Processing of Alzheimer β /A4 amyloid precursor protein: Modulation by agents that regulate protein phosphorylation. *Proc. Natl. Acad. Sci. USA*, **87**, 6003–6006.
- Buxbaum, J.D., Koo, E.H., and Greengard, P. (1993) Protein phosphorylation inhibits production of Alzheimer amyloid β /A4 peptide. *Proc. Natl. Acad. Sci. USA*, **90**, 9195–9198.
- Capell, A., Safrich, R., Olivo, J.-C., Meyn, L., Walter, J., Grünberg, J., Methews, P., Nixon, R., Dotti, C., and Haass C. (1997) Cellular expression and proteolytic processing of presenilin proteins is developmentally regulated during neuronal differentiation. *J. Neurochem.*, **69**, 2432–2440.
- Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T., and Hidaka, H. (1990) Inhibition of forskolin induced neurite outgrowth and protein phosphorylation by a newly selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC-12D pheochromocytoma cells. *J. Biol. Chem.*, **265**, 5267–5272.
- Cohen, P. (1992) Signal transduction at the level of protein kinases, protein phosphatases and their substrates. *Trends Biochem. Sci.*, **17**, 408–413.
- Cook, D.G., Sung, J.C., Golde, T.E., Felsenstein, K.M., Wojczyk, B.S., Tanzi, R.E., Trojanowski, J.Q., Lee, V.M.-Y., and Doms, R.W. (1996) Expression and analysis of presenilin 1 in a human neuronal system: Localization in cell bodies and dendrites. *Proc. Natl. Acad. Sci. USA*, **93**, 9223–9228.

- De Strooper, B., Beullens, M., Contreras, B., Levesque, L., Craessaerts, K., Cordell, B., Moechars, D., Bollen, M., Fraser, P., St. George-Hyslop, P., George-Hyslop, P.M. and van Leuven, F. (1997) Phosphorylation, subcellular localization and membrane orientation of the Alzheimer's disease-associated presenilins. *J. Biol. Chem.*, **272**, 3590–3598.
- Deng, G., Pike, C.J., and Cotman, C.W. (1996) Alzheimer-associated presenilin-2 confers increased sensitivity to apoptosis in PC 12 cells. *FEBS Lett.*, **397**, 50–54.
- Doan, A., Thinakaran, G., Borchelt, D.R., Slunt, H.H., Ratovitsky, T., Podlisny, M., Selkoe, D.J., Seeger, M., Gandy, S.E., Price, D.L., and Sisodia, S.S. (1996) Protein topology of presenilin 1. *Neuron*, **17**, 1023–1030.
- Goedert, M. (1993) Tau protein and the neurofibrillary pathology of Alzheimer's disease. *Trends Neurosci.*, **16**, 460–465.
- Goedert, M., Spillantini, M.G., Cairns, N.J., and Crowther, R.A. (1992) Abnormal tau phosphorylation at serine396 in Alzheimer's disease recapitulates development and contributes to reduced microtubule binding. *Neuron*, **8**, 159–168.
- Grünberg, J., Walter, J., Loetscher, H., Deuschle, U., Jacobson, H., and Haass, C. (1998) The Alzheimer's disease associated presenilin-1 holoprotein and its 18–20 kDa C-terminal fragment are death substrates for proteases of the caspase family. *Biochemistry*, **37**, 2263–2270.
- Grundke-Iqbal, I., Iqbal, K., Tung, Y.-C., Quinlan, M., Wisniewski, H.M., and Binder, L.I. (1986) Abnormal phosphorylation of the microtubule-associated protein τ (tau) in Alzheimer cytoskeletal pathology. *Proc. Natl. Acad. Sci.*, **83**, 4913–4917.
- Hartmann, H., Busciglio, J., Baumann, K.-H., Staufenbiel, M., and Yankner, B.A. (1997) Developmental regulation of presenilin-1 processing in the brain suggests a role in neuronal differentiation. *J. Biol. Chem.*, **272**, 14505–14508.
- Hung, A.Y., and Selkoe, D.J. (1994) Selective ectodomain phosphorylation and regulated cleavage of β -amyloid precursor protein. *EMBO J.*, **13**, 534–542.
- Hung, A.Y., Haass, C., Nitsch, R.M., Qiu, W.Q., Citron, M., Wurtman, R.J., and Selkoe, D.J. (1993) Activation of protein kinase C inhibits cellular production of the amyloid β -protein. *J. Biol. Chem.*, **268**, 22959–22962.
- Hunter, T. (1995) Protein kinases and Phosphatases: The Yin and Yang of protein phosphorylation and signaling. *Cell*, **80**, 225–236.
- Kim, T.-W., Pettingell, W.H., Hallmark, O.G., Moir, R.D., Wasco, W., and Tanzi, R.E. (1997a) Endoproteolytic cleavage and proteasomal degradation of presenilin 2 in transfected cells. *J. Biol. Chem.*, **272**, 11006–11010.
- Kim, T.-W., Pettingell, W.H., Jung, Y.-K., Kovacs, D.M., and Tanzi, R.E. (1997b) Alternative cleavage of Alzheimer-associated presenilins during apoptosis by a caspase-3 family member. *Science*, **277**, 373–376.
- Knops, J., Gandy, S., Greengard, P., Lieberburg, L., and Sinha, S. (1993) Serine phosphorylation of the secreted extracellular domain of APP. *Biochem. Biophys. Res. Comm.*, **197**, 380–385.
- Kovacs, D.M., Fausett, H.J., Page, K.J., Kim, T.-W., Moir, R.D., Merriam, D.E., Hollister, R.D., Hallmark, O.G., Mancini, R., Felsenstein, K.M., Hyman, B.T., Tanzi, R.E., and Wasco, W. (1996) Alzheimer-associated presenilins 1 and 2: Neuronal expression in brain and localization to intracellular membranes in mammalian cells. *Nature Med.*, **2**, 224–229.
- Krebs, E.G. (1993) Protein phosphorylation and cellular regulation. *Biosci. Reports*, **13**, 127–142.
- Lee, M.K., Borchelt, D.R., Kim, G., Thinakaran, G., Slunt, H.H., Ratovitski, T., Martin, L.J., Kittur, A., Gandy, S., Levey, A.I., Jenkins, N., Copeland, N., Price, D.L., and Sisodia, S.S. (1997) Hyperaccumulation of FAD-linked presenilin 1 variants *in vivo*. *Nature Med.*, **3**, 756–760.

- Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D.M., Oshima, J., Pettingell, W.H., Yu, C.-E., Jondro, P.D., Schmidt, S.D., Wang, K., Crowley, A.C., Fu, Y.-H., Guenette, S.Y., Galas, D., Nemes, E., Wijsman, E.M., Bird, T.D., Schellenberg, G.D., and Tanzi, R.E. (1995) Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science*, **269**, 973–977.
- Li, X., and Greenwald, I. (1996) Membrane topology of the *C. elegans* SEL-12 presenilin. *Neuron*, **17**, 1015–1021.
- Lin, R., Beuparlant, P., Makris, C., Meloche, S., and Hiscott J. (1996) Phosphorylation of I κ B α in the C-terminal PEST domain by casein kinase II affects intrinsic protein stability. *Mol. Cell. Biol.*, **16**, 1401–1409.
- Loetscher, H., Deuschle, U., Brockhaus, M., Reinhardt, D., Nelboeck, M., Grünberg, J., Haass, C., and Jacobsen, H. (1997). Presenilins are processed by caspase-type proteases. *J. Biol. Chem.*, **272**, 20655–20659.
- MacKichan, M., Logeat, F., and Israël, A. (1996) Phosphorylation of p105 PEST sequences via a redox-insensitive pathway up-regulates processing to p50 NF- κ B. *J. Biol. Chem.*, **271**, 6084–6091.
- Mercken, M., Takahashi, H., Honda, T., Sato, K., Murayama, M., Nakazato, Y., Noguchi, K., Imahori, K., and Takashima, A. (1996) Characterization of human presenilin 1 using N-terminal specific monoclonal antibodies: Evidence that Alzheimer mutations affect proteolytic processing. *FEBS Lett.*, **389**, 297–303.
- Oishi, M., Nairn, A.C., Czernik, A.J., Lim, G.S., Isohara, T., Gandy, S.E., Greengard, P., and Suzuki, T. (1997) The cytoplasmic domain of Alzheimer's amyloid precursor protein is phosphorylated at Thr654, Ser655, and Thr668 in adult rat brain and cultured cells. *Mol. Med.*, **3**, 111–123.
- Pearson, R.B., and Kemp, B.E. (1991) Protein kinase phosphorylation site sequences and consensus specificity motifs: Tabulations. *Meth. Enzymol.*, **200**, 62–81.
- Perez-Tur, J., Froelich, S., Prihar, G., Crook, R., Baker, M., Duff, K., Wragg, M., Busfield, F., Lendon, C., Clark, R.F., Roques, P., Fulder, R.A., Johnston, J., Cowburn, R., Forsell, C., Axelman, K., Lilius, L., Houlden, H., Karran, E., Roberts, G.W., Rossor, M., Adams, M.D., Hardy, J., Goate, A., Lannfelt, L., and Hutton, M. (1995) A mutation in Alzheimer's disease destroying a splice acceptor site in the presenilin-1 gene. *Neuroreport*, **7**, 297–301.
- Podlisny, M.B., Citron, M., Amarante, P., Sherrington, R., Xia, W., Zhang, J., Diehl, T., Levesque, G., Fraser, P., Haass, C., Koo, E.H., Seubert, P., St. George-Hyslop, P.H., Teplow, D.B., and Selkoe, D.J. (1997) Presenilin proteins undergo heterogeneous endoproteolysis between Thr291 and Ala299 and occur as stable N- and C-terminal fragments in normal and Alzheimer brain tissue. *Neurobiol. Dis.*, **3**, 325–337.
- Rechsteiner, M., and Rogers, S.W. (1996) PEST sequences and regulation by proteolysis. *Trends Biochem. Sci.*, **21**, 267–271.
- Rogaev, E.I., Sherrington, R., Rogaeva, E.A., Levesque, G., Ikeda, M., Liang, Y., Chi, H., Lin, C., Holman, K., Tsuda, T., Mar, L., Sorbi, S., Nacmias, B., Piacentini, S., Amaducci, L., Chumakov, I., Cohen, D., Lannfelt, L., Fraser, P.E., Rommens, J.M., and St. George-Hyslop, P.H. (1995) Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to Alzheimer's disease type 3 gene. *Nature*, **376**, 775–778.
- Seeger, M., Nordstedt, C., Petanceska, S., Kovacs, D.M., Gouras, G.K., Hahne, S., Fraser, P., Levesque, L., Czernik, A.J., St. George-Hyslop, P.H., Sisodia, S.S., Thinakaran, G., Tanzi, R.E., Greengard, P., and Gandy, S. (1997) Evidence for phosphorylation and oligomeric assembly of presenilin 1. *Proc. Natl. Acad. Sci. USA*, **94**, 5090–5094.
- Suzuki, T., Oishi, M., Marshak, D.R., Czernik, A.J., Nairn, A.C., and Greengard, P. (1994) Cell cycle-dependent regulation of the phosphorylation and metabolism of the Alzheimer amyloid precursor protein. *EMBO J.*, **13**, 1114–1122.

- Thinakaran, G., Borchelt, D.R., Lee, M.K., Slunt, H.H., Spitzer, L., Kim, G., Ratovitsky, T., Davenport, F., Nordstedt, C., Seeger, M., Hardy, J., Levey, A.I., Gandy, S.E., Jenkins, N.A., Copeland, N.G., Price, D.L., and Sisodia, S.S. (1996) Endoproteolysis of presenilin 1 and accumulation of processed derivatives *in vivo*. *Neuron*, **17**, 181–190.
- Tomita, T., Maruyama, K., Takaomi, C.S., Kume, H., Shinozaki, K., Tokuhiro, S., Capell, A., Walter, J., Grünberg, J., Haass, G., Iwatsubo, T., and Obata, K. (1997) The presenilin 2 mutation (N141I) linked to familial Alzheimer disease (Volga German families) increases the secretion of amyloid β protein ending at the 42nd (or 43rd) residue. *Proc. Natl Acad. Sci.*, **94**, 2025–2030.
- Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle *et al.*, (1991) The bisindolylmaleimide GF109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.*, **266**, 15771–15781.
- Trojanowski, J.Q., and Lee, V.M. (1995) Phosphorylation of paired helical filament tau in Alzheimer's disease neurofibrillary lesions: focusing on phosphatases. *FASEB J.*, **9**, 1570–1576.
- Vito, P., Lacan , E., and D'Adamio, L. (1996a) Interfering with apoptosis: Ca^{2+} -binding protein ALG-2 and Alzheimer's disease gene ALG-3. *Science*, **271**, 521–525.
- Vito, P., Wolozin, B., Ganjei, J.K., Iwasaki, K., Lacan , E., and D'Adamio, L. (1996b) Requirement of the familial Alzheimer's disease gene PS2 for apoptosis. *J. Biol. Chem.*, **271**, 31025–31028.
- Walsh, D.A., and van Patten, S.M. (1994) Multiple pathway signal transduction by the cAMP-dependent protein kinase. *FASEB J.*, **8**, 1227–1236.
- Walter, J., Capell, A., Gr nberg, J., Pesold, B., Schindzielorz, A., Prior, R., Podlisny, M.B., Fraser, P., St. George-Hyslop, P.H., Selkoe, D.J., and Haass, C. (1996) The Alzheimer's disease-associated presenilins are differentially phosphorylated proteins located predominantly within the endoplasmic reticulum. *Mol. Med.*, **2**, 673–691.
- Walter, J., Capell, A., Hung A.Y., Langen, H., Schn lzer, M., Thinakaran, G., Sisodia, S.S., Selkoe, D.J., and Haass, C. (1997a) Ectodomain phosphorylation of β -amyloid precursor protein at two distinct cellular locations. *J. Biol. Chem.*, **272**, 1896–1903.
- Walter, J., Gr nberg, J., Capell, A., Pesold, B., Schindzielorz, A., Citron, M., Mendla, K., St. George-Hyslop, P., Multhaup, G., Selkoe, D.J., and Haass, C. (1997b) Proteolytic processing of the Alzheimer disease-associated presenilin-1 generates an *in vivo* substrate for protein kinase C. *Proc. Natl. Acad. Sci. USA*, **94**, 5349–5354.
- Walter, J., Gr nberg, J., Schindzielorz, A. and Haass, C. (1998) Proteolytic processing products of Presenilin-1 and -2 are phosphorylated *in vivo* by distinct cellular mechanisms. *Biochemistry*, **37**, 5961–5967.
- Wera, S., and Hemmings, B.A (1995) Serine/threonine protein phosphatases. *Biochem. J.*, **311**, 17–29.
- Wolozin, B., Iwasaki, K., Vito, P., Ganjei, J.K., Lacan , E., Sunderland, T., Zhao, B., Kusiak, J.W., Wasco, W., and D'Adamio, L. (1996) Participation of presenilin 2 in apoptosis: Enhanced basal activity conferred by an Alzheimer mutation. *Science*, **274**, 1710–1713.

ROLE OF RISK FACTORS IN ALZHEIMER'S DISEASE

16.

APOE AND ITS ROLE IN LATE ONSET ALZHEIMER'S DISEASE

G.WILLIAM REBECK

*Alzheimer's Research Center, Massachusetts General Hospital, 149 13th St Charlestown,
MA 02129, USA*

The apolipoprotein E gene (APOE) exists in three major alleles: APOE- ϵ 2, APOE- ϵ 3, and APOE- ϵ 4. The frequency of these alleles in Caucasian populations is: APOE- ϵ 2, 0.08; APOE- ϵ 3, 0.78; APOE- ϵ 4, 0.14 (Davignon *et al.*, 1988; Ordovas *et al.*, 1987). The apoE isoforms of these allelic variants have different arginine/cysteine compositions:

<i>allele</i>	<i>frequency</i>	<i>isoform</i>		
APOE- ϵ 2:	0.08	apoE2	Cys 112	Cys 158
APOE- ϵ 3	0.78	apoE3	Cys 112	Arg 158 +
APOE- ϵ 4	0.14	apoE4	Arg 112+	Arg 158+

Thus, the three isoforms differ in their charge (Zannis and Breslow, 1981) and in the presence of two, one, or zero cysteine residues.

The product of the APOE gene is a 299 amino acid protein (apoE) found associated with lipid particles in the plasma and the CSF (Mahley, 1988). ApoE has two major functional domains: an N-terminal receptor binding domain, and a C-terminal lipid binding domain (Mahley, 1988). The altered amino acids, 112 and 158, are in the receptor binding domain. ApoE2 has dramatically decreased affinity for receptor binding (Weisgraber *et al.*, 1982), but there have been no reports of differences between apoE3 and apoE4 for receptor binding. The two domains of apoE interact (Weisgraber, 1994), and apoE2 and apoE4 have altered interactions with lipid particles compared to apoE3 (Gregg *et al.*, 1986; Weisgraber, 1990).

GENETICS OF APOE AND ALZHEIMER'S DISEASE

Analysis of late onset familial Alzheimer's disease (AD) indicated that a gene on chromosome 19 acted as a genetic risk factor for AD (Pericak-Vance *et al.*, 1991). APOE was identified as a candidate gene when it was found associated with A β (Strittmatter *et al.*, 1993) and amyloid plaques (Namba *et al.*, 1991). The APOE- ϵ 4 allele was over-represented in AD patients (0.52 in AD vs. 0.16 in controls

(Strittmatter *et al.*, 1993)), a finding that was replicated in numerous studies of familial and sporadic AD (Chartier-Harlin *et al.*, 1994; Czech *et al.*, 1993; Mayeux *et al.*, 1993; Poirier *et al.*, 1993; Rebeck *et al.*, 1993; Saunders *et al.*, 1993; Tsai *et al.*, 1994) (for reviews, see (Strittmatter and Roses, 1996; van Gool *et al.*, 1995)). While APOE- ϵ 4 was over-represented three to four-fold in AD, APOE- ϵ 2 was under-represented: the APOE- ϵ 2 allele frequency ranged from 0.01 to 0.04 in AD populations, compared to 0.08 in control populations (Benjamin *et al.*, 1994; Corder *et al.*, 1994; Gomez-Isla *et al.*, 1996; Talbot *et al.*, 1994; West *et al.*, 1994). Together these data argue that it is the changes in the APOE gene itself responsible for the altered risk of AD, and not merely linkage of the APOE- ϵ 4 and APOE- ϵ 2 alleles with other mutations.

This association of APOE alleles with altered risks of AD was also found in population-based epidemiologic studies. Analysis of aged individuals in Iowa (Hyman *et al.*, 1996) and Australia (Henderson *et al.*, 1995) demonstrated that inheritance of APOE- ϵ 4 was associated with a 1.4 to 1.9-fold increased risk of dementia over four years of follow-up study. Interestingly, many APOE- ϵ 4/4 individuals were identified in both populations who had lived well beyond the age of 80 without signs of AD, further emphasizing the designation of APOE- ϵ 4 only as a risk factor for AD. In agreement with the studies of AD, analysis of normal aging showed that APOE- ϵ 4 was under-represented in aged individuals (Rebeck *et al.*, 1994), and APOE- ϵ 2 was over-represented in aged individuals (Schachter *et al.*, 1994).

An effect of APOE genotype on other neurodegenerative diseases remains unproven. APOE- ϵ 4 allele frequency is not increased in amyelolateral sclerosis (ALS) (Moulard *et al.*, 1996; Mui *et al.*, 1995), Creutzfeldt-Jakob disease (Nakagawa *et al.*, 1995; Saunders *et al.*, 1993; Zerr *et al.*, 1995), Pick's disease (Fairer *et al.*, 1995; Gomez-Isla *et al.*, 1996; Minthon *et al.*, 1997; Pickering-Brown *et al.*, 1995), Parkinson's disease (Benjamin *et al.*, 1994; Harrington *et al.*, 1994; Koller *et al.*, 1995), Huntington's chorea (Harrington *et al.*, 1994), or progressive supranuclear palsy (Gomez-Isla *et al.*, 1996). However, it is possible that APOE genotype affects the age of onset, or rate of progression of these diseases. For example, in ALS, one study found that APOE- ϵ 4 affected the risk of bulbar-onset disease (A1-Chalabi *et al.*, 1996), and another study found that APOE- ϵ 2 individuals had a longer median survival (Moulard *et al.*, 1996). In Cruetzfeldt-Jakob disease, APOE- ϵ 2 was associated with later age of onset (Pickering-Brown *et al.*, 1995) and delayed death (Amouyel *et al.*, 1994), although this association was not seen in other studies (Nakagawa *et al.*, 1995; Salvatore *et al.*, 1995; Saunders *et al.*, 1993). In Pick's disease, two studies showed an association between APOE- ϵ 4 and earlier ages of onset (Farrer *et al.*, 1995; Minthon *et al.*, 1997). In Parkinson's disease, APOE genotype showed no effect on the extent of neuropathological changes (Gearing *et al.*, 1995). In Huntington's disease, APOE genotype did not affect age of onset (Rubinsztein *et al.*, 1997). These are important studies when generating models of apoE's involvement in AD: does apoE affect some AD-specific pathological process, or does apoE affect some process common to many neurodegenerative diseases? So far the data support a role in AD-specific changes; however, in-depth analysis of other disease may reveal subtle roles for apoE.

NORMAL EXPRESSION AND FUNCTIONS OF APOE IN THE CNS

In the CNS, apoE is expressed in astrocytes (Boyles *et al.*, 1985; Pitas *et al.*, 1987; Poirier *et al.*, 1991). In culture, apoE produced by astrocytes is released in association with lipids (LaDu *et al.*, 1998), forming small, high density lipoproteins. The particles presumably are the precursors of the lipoproteins found in the CSF, although small amounts of plasma lipoproteins may cross the blood brain barrier (Pitas *et al.*, 1987; Segal, 1993).

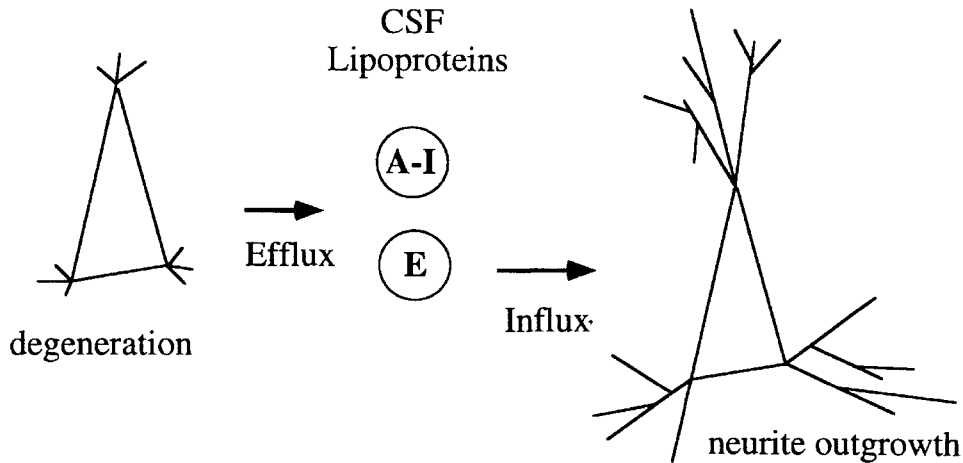


Figure 1. Roles for CSF lipoproteins in cholesterol efflux and influx.

There are at least three main populations of lipoproteins in the CSF. ApoE is associated with high density lipoproteins (HDL) which are primarily spherical, and 14 to 20 nm in size (Borghini *et al.*, 1995; Pitas *et al.*, 1987). A second class of high density lipoproteins contains primarily apoA-I (Borghini *et al.*, 1995; Pitas *et al.*, 1987). A third class of lipoprotein is larger (34 nm), and contains neither apoE or apoA-I (Borghini *et al.*, 1995). There is further complexity associated with these three populations of lipoproteins, with many other apolipoproteins present in the CSF, including apoA-II, apoA-IV, apoC-I, apoCII, apoC-III, apoD, and apoJ (Borghini *et al.*, 1995; Koudinov *et al.*, 1996; Pitas *et al.*, 1987; Roheim *et al.*, 1979).

There are two main functions of lipoproteins: removal of lipids from loaded cells, and delivery of lipids to starved cells. In the plasma, HDL are used for the removal of lipids from cells. Plasma HDL contain primarily apoA-I and apoA-II; a small fraction of plasma HDL contains only apoE, and is termed γ -LpE (Huang *et al.*, 1994). Both of these types of HDL efficiently remove cholesterol from cells. CSF lipoproteins also remove cholesterol from loaded cells in culture (Rebeck *et al.*, 1998), probably via both the apoA-I and the apoE containing lipoproteins. The transfer of lipids to lipoproteins presumably changes the composition of the particles, leading to a larger, less dense lipoprotein (Figure 1).

In the plasma, delivery of lipids to cells occurs mainly via larger lipoproteins, such as low density lipoproteins (LDL) and very low density lipoproteins (VLDL), but it can also occur via HDL. Delivery of lipids to cells occurs primarily through the interactions of apoE and apoB with cell surface receptors.

Since there is no apoB in the CSF, uptake of lipoproteins in the CNS is presumably primarily mediated by apoE (Figure 1).

In fibroblasts and hepatocytes, the initial binding of apoE-containing lipoproteins occurs via heparan sulfate proteoglycans (Ji *et al.*, 1993). ApoE secreted from cells remains associated with cell surface proteoglycans, and this apoE aids in the binding of exogenous lipoproteins (Ji *et al.*, 1994). It is likely that a similar situation occurs in the CNS with secretion of apoE by astrocytes. After binding to the cell surface, the apoE-containing lipoprotein is then transferred to a second binding site, and the receptor-ligand complex is internalized and directed to the endosomal/lysosomal pathway (Weisgraber, 1994).

Uptake of CSF lipoproteins could occur via several receptors found in the CNS. CSF lipoproteins can block binding of LDL to the LDL receptor (Pitas *et al.*, 1987), suggesting that the LDL receptor is one route lipids into cells. *In situ* hybridization (Swanson *et al.*, 1988) and immunohistochemistry (Pitas *et al.*, 1987; Rebeck *et al.*, 1993) suggest that the LDL receptor is expressed in low levels throughout the brain. But there are several other receptors related to the LDL receptor which could mediate uptake of CSF lipoproteins. LRP is present on neurons, activated astrocytes, and choroid plexus (Moestrup *et al.*, 1992; Rebeck *et al.*, 1993; Tooyama *et al.*, 1993; Wolf *et al.*, 1992). The VLDL receptor is present on microglia (Christie *et al.*, 1996). The apoE receptor-2 is primarily expressed in brain, and present in several brain regions (Kim *et al.*, 1996). Megalin (GP330) is present on ependymal cells (Zheng *et al.*, 1994). Each of these receptors could mediate uptake of CSF lipoproteins into distinct subsets of CNS cells, or under distinct conditions. For example, LRP binds CSF lipoproteins only in the presence of excess amounts of apoE (Bellosta *et al.*, 1995; Fagan *et al.*, 1996). Thus, under normal conditions, it appears that CSF lipoproteins can be taken up by an LDL receptor-mediated mechanism, but when apoE expression is induced, CSF lipoproteins may be directed toward LRP.

There is another class of receptors which could mediate CSF lipoprotein uptake, the scavenger receptors. These receptors bind oxidized and acetylated forms of lipoproteins (Krieger and Herz, 1994). One member of this family, scavenger receptor A, is present on microglia (Christie *et al.*, 1996) and is particularly interesting in AD because it also mediates uptake of the A β peptide by microglia (El Khoury *et al.*, 1996; Paresce *et al.*, 1996). Perhaps under some conditions, such as severe oxidative stress, CSF lipoproteins are cleared via scavenger receptors, a mechanism which could affect clearance of A β .

EXPRESSION AND FUNCTIONS OF APOE IN AD

As noted above, genetic analyses demonstrated that inheritance of APOE- ϵ 4 is associated with an increased risk of AD and an earlier age of onset, and inheritance of APOE- ϵ 2 is associated with a decreased risk of AD and a later age of onset. Immunohistochemical analyses found that the apoE protein is associated with both amyloid deposits and NFT in the AD brain (Benzing and Mufson, 1995; Namba *et al.*, 1991; Rebeck *et al.*, 1993; Schmechel *et al.*, 1993; Wisniewski and Frangione, 1992). APOE- ϵ 4 was associated with increased levels of amyloid (Gomez-Isla *et al.*, 1996; Harrington *et al.*, 1994; Nagy *et al.*, 1995; Ohm *et al.*, 1995; Polvikoski *et al.*, 1995; Rebeck *et al.*, 1993; Schmechel *et al.*, 1993) and APOE- ϵ 2 was associated with decreased amyloid (Polvikoski *et al.*,

1995). Similar findings were reported for other conditions with A β deposition, Down's syndrome (Hyman *et al.*, 1995), cerebral amyloid angiopathy (Greenberg *et al.*, 1995; Premkumar *et al.*, 1996), and head trauma (Nicoll *et al.*, 1995). APOE- ϵ 4 was not associated with increased rate of NFT formation (Gomez-Isla *et al.*, 1996; Schmechel *et al.*, 1993) or the loss of neurons (Gomez-Isla *et al.*, 1997).

Several hypotheses have been generated to explain how the function of apoE4 may affect the pathobiology of AD. First, apoE4 may be catabolized at a different rate than apoE3 in the CNS, affecting its ability to repair neuronal damage. Second, the delivery of lipoproteins to neurons by apoE4 may not protect neurons from degeneration as well as apoE3, leading to NFT or cell loss. Third, removal of lipophilic debris, such as A β , by apoE4 may be less efficient than by apoE3, resulting in accumulation of amyloid. There are of course other hypotheses, but this review will focus on data concerning these three.

Levels of apoE in the CNS

In models of optic and peripheral nerve degeneration, both apoE (Boyles *et al.*, 1990; Dawson *et al.*, 1986; Ignatius *et al.*, 1986; Snipes *et al.*, 1986) and apoA-I (Boyles *et al.*, 1990; Dawson *et al.*, 1986; Harel *et al.*, 1989) are induced dramatically. The increased levels of these apolipoproteins facilitates the removal of excess cholesterol (Goodrum, 1991). In models of acute neuronal degeneration in the CNS, apoE is also transiently induced (Poirier *et al.*, 1991). There is a later decrease in HMG-CoA reductase, an enzyme necessary for the *in situ* production of cholesterol and an increase in LDL binding (Poirier *et al.*, 1993). These data support a model of decreased cholesterol synthesis and increased cholesterol scavenging in the CNS after acute damage (Poirier *et al.*, 1993).

One might have expected increased apoE levels in chronic neurodegeneration, such as AD. However, in studies of CSF apoE levels in AD, Blennow found a 70% decrease in CSF apoE levels (Blennow *et al.*, 1994), and Skoog reported a 32% decrease in CSF apoE levels with AD in 85 year olds (Skoog *et al.*, 1997). Several other studies reported no change in CSF apoE levels in AD (Chauhan *et al.*, 1996; Hahne *et al.*, 1997; Lefranc *et al.*, 1996; Roesler *et al.*, 1996), although one study found a 2.4-fold increase of CSF apoE in AD (Merched *et al.*, 1997). No changes were reported for several other neurodegenerative diseases, including vascular dementia (Pirtilla *et al.*, 1996), CreutzfeldtJakob disease (Zerr *et al.*, 1996), or non-specified neurological disorders (Carlsson *et al.*, 1991; Lefranc *et al.*, 1996; Roesler *et al.*, 1996). Thus, unlike in acute neurodegeneration, there is not a dramatic increase in apoE in chronic neurodegeneration. Inheritance of APOE ϵ 4 was also not associated with a change in CSF apoE levels (Chauhan *et al.*, 1996; Lefranc *et al.*, 1996; Merched *et al.*, 1997). There is, however, one report that apoE levels in the brains of APOE- ϵ 4 AD patients are decreased (Bertrand *et al.*, 1995) (although another study found no difference (Harr *et al.*, 1996)), raising the possibility that apoE4 in the neuropil is produced or degraded at a different rate than apoE3.

Effects of apoE on Neuronal Cells

The effects of apoE-containing lipids on neuronal cells have been examined using dorsal root ganglion cells (Handelmann *et al.*, 1992; Nathan *et al.*, 1994), primary hippocampal cells (Narita *et al.*, 1997;

Puttfarcken *et al.*, 1997), and neuronal cell lines (Bellosta *et al.*, 1995; Fagan *et al.*, 1996; Holtzman *et al.*, 1995; Nathan *et al.*, 1995). A consistent finding of these studies is that apoE3 promotes neurite outgrowth, leading to longer neurites and more neurite branching. ApoE4 does not promote neurite outgrowth as effectively, and in some assays actually prevents neurite outgrowth. These effects are inhibited by blocking LRP function (Bellosta *et al.*, 1995; Fagan *et al.*, 1996; Holtzman *et al.*, 1995; Narita *et al.*, 1997), suggesting that LRP mediates the effects of apoE on neurite outgrowth in CNS-derived cells. Another LRP ligand, α 2-macroglobulin, also acts to promote neurite outgrowth (Mori *et al.*, 1990). These data raise the interesting possibility that LRP may act not only as an endocytic receptor, but may also be involved in signal transduction pathways.

One puzzling finding is the apparent accumulation of apoE in the cytoplasm of neurons. Internalization of LRP directs receptor-ligand complexes to the endosomal/lysosomal pathway, where ligands are released from LRP and degraded, while LRP recycles to the cell membrane (Herz, 1993). Thus, by immunohistochemistry, one would expect to observe only low levels of apoE in intracellular vesicles. However, there are several reports of strong apoE immunoreactivity in neurons (Benzing and Mufson, 1995; Han *et al.*, 1994; Han *et al.*, 1994; Metzger *et al.*, 1996), suggesting that apoE may be transferred from intracellular vesicles to the cytoplasm. Based on *in vitro* studies, it has been suggested that cytoplasmic apoE interacts with microtubules and provides stabilization, with apoE3 binding more avidly than apoE4 (Strittmatter *et al.*, 1994). Treatment of Neuro-2a cells with apoE-lipoproteins led to an accumulation of apoE in cell bodies and neurites, an accumulation that was not evident in fibroblasts (Nathan *et al.*, 1995). Furthermore, more apoE accumulated and more tubulin was in a polymerized state in apoE3-treated cells than in cells treated with apoE4. It remains to be determined whether all neurons accumulate apoE, or whether there is something particular to the neurons that are susceptible in Alzheimer's disease.

Interactions Between apoE and Amyloid

There are two main hypotheses concerning the potential effect of apoE on amyloid. The first is that apoE affects the aggregation of A β into fibrils, and the second is that apoE facilitates A β clearance from the CSF or neuropil. Both of these models depend on defining an interaction between apoE and A β . Strittmatter (Strittmatter *et al.*, 1993) first showed that purified apoE interacted with A β , with the lipid binding domain of apoE binding to A β . In a more physiologically relevant form, apoE on lipoproteins also binds A β , with apoE3 showing increased affinity compared to apoE4 (LaDu *et al.*, 1994; LaDu *et al.*, 1995). Furthermore, A β was found associated with CSF lipoproteins (Koudinov *et al.*, 1996), providing evidence that an apoE-A β interaction may be occurring *in vivo*.

An effect of apoE on A β deposition. How soluble A β undergoes the transition to deposited A β remains one of the central questions of AD. One hypothesis is that the conformation of A β is altered as it associates with other proteins, and once in the new conformation, it can act as a seed for further A β aggregation (Wisniewski and Frangione, 1992). There are several A β binding proteins identified in CSF: transthyretin (Schwarzman *et al.*, 1994), apoJ (Ghisso *et al.*, 1993), and apoE (Strittmatter *et al.*, 1993), which could promote or prevent A β aggregation *in vivo*. Several *in vitro* assays have shown that purified apoE can promote A β aggregation (Castano *et al.*, 1995; Ma *et al.*, 1994; Sanan *et al.*, 1994), with apoE4 more potent than apoE3 and apoE2 at facilitating fibrillogenesis. Conversely, other reports

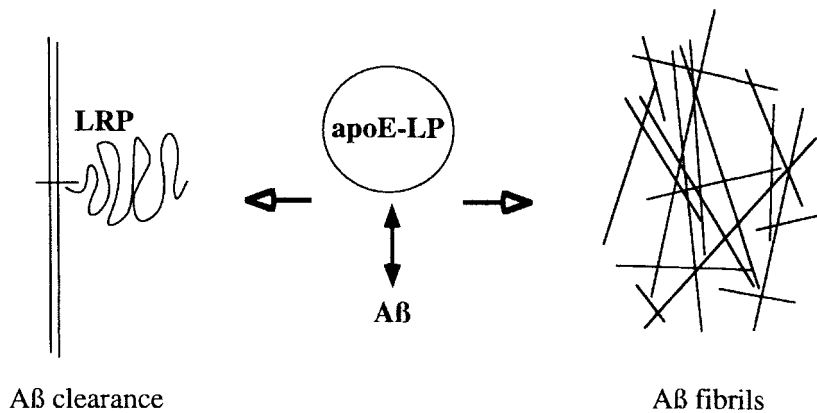


Figure 2. Effects of the interaction between A β and apoE-containing lipoproteins.

indicate that apoE can prevent A β aggregation (Evans *et al.*, 1995), with apoE4 less potent than apoE3. So far, it is unknown how apoE associated with brain lipoproteins affects A β aggregation.

An effect of apoE on A β clearance. Models of amyloid in AD suggest that there is a dynamic balance of amyloid deposition and amyloid clearance in the AD brain (Cruz *et al.*, 1997; Hyman *et al.*, 1993). These models are supported by *in vivo* findings that amyloid does not accumulate with increased duration of disease (Arriagada *et al.*, 1992; Gomez-Isla *et al.*, 1996) and by *in vitro* findings that A β can be removed by cells in culture (El Khoury *et al.*, 1996; Ida *et al.*, 1996; Paresce *et al.*, 1996; Shaffer *et al.*, 1995; Yan *et al.*, 1996). Some of this removal seems to be direct, through binding of A β to Scavenger receptor A (El Khoury *et al.*, 1996; Paresce *et al.*, 1996) or the receptor for advanced glycation end products (Yan *et al.*, 1996) on microglia. Other removal may depend on A β first complexing with other molecules, such as apoE on lipoproteins (LaDu *et al.*, 1994) or α 2-macroglobulin (Du *et al.*, 1997), which could then be cleared by receptors such as LRP. Indeed, binding of A β by apoE3 was more avid than binding by apoE4, supporting a model of decreased A β clearance by apoE4 (LaDu *et al.*, 1994).

Of course, there could be a balance between apoE facilitating aggregation of A β and apoE facilitating clearance of apoE (Figure 2). A change in this balance in between apoE isoforms could lead to more amyloid deposition with apoE4 and less with apoE2. Changes in this balance could also affect the toxicity of A β . Recently LaDu and colleagues have found that apoE3-lipoproteins reduce A β -induced neuronal toxicity by facilitating an increased removal of A β via LRP (Puttfarcken *et al.*, 1997; Jordon *et al.*, 1998).

A CRITICAL ROLE FOR LRP IN AD?

Understanding the role of apoE in AD naturally requires study of apoE receptors. One of the most surprising findings of the research of apoE in AD is that LRP acts as a receptor for both apoE and some forms of the amyloid precursor protein, APP (Kounnas *et al.*, 1995). LRP is a multifunctional receptor, acting to bind and clear numerous molecules including lipoprotein-associated molecules

(apoE, lipoprotein lipase, hepatic lipase), proteinase/proteinase inhibitor complexes (plasminogen activators, plasminogen activator inhibitor-1, tissue factor pathway inhibitor, α 2-macroglobulin), and other molecules (lactoferrin, thrombospondin) (Herz, 1993). Kunitz protease inhibitor-containing forms of APP are also LRP ligands (Kounnas *et al.*, 1995); while this was first shown for soluble forms of APP, it is also true of membrane bound forms of APP, as shown by studies of turnover of cell surface APP (Knauer *et al.*, 1996), and immunoprecipitation experiments (unpublished data). Thus, apoE may be linked to AD via an effect on APP metabolism.

In addition to the work on LRP mediating apoE-induced neurite outgrowth (described above), and LRP clearing apoE-A β complexes (also described above), there are several other interesting links between LRP and AD. LRP and many of its ligands are found on amyloid deposits in AD, suggesting that there is impaired LRP activity in the AD brain (Rebeck *et al.*, 1995). One ligand of LRP, α 2-macroglobulin, interacts *in vitro* with A β (Du *et al.*, 1997) and with a protease that degrades A β (Qiu *et al.*, 1996). Finally, an LRP polymorphism is linked to increased risk of AD, an association that is age-dependent, and that affects amyloid deposition (Kang *et al.*, 1997), similar to APOE. Whether these observations are critical in the pathogenesis of AD is unknown. However, an understanding of the roles of apoE and APP in the CNS depends on an understanding of their receptor LRP, and the potential dysfunction of LRP-mediated clearance in AD.

REFERENCES

- Al-Chalabi, A., Enayat, Z.E., Bakker, M.C., Sham, P.C., Ball, D.M., Shaw, C.E., Lloyd, C.M., Powell, J.F., and Leigh, P.N. (1996). Association of apolipoprotein E ϵ 4 with bulbar onset motor neuron disease. *Lancet*, **347**, 159–160.
- Amouyel, P., Vidai, O., Launey, J.M., and Laplanche, J.L. (1994). The apolipoprotein E alleles as major susceptibility factors for Creutzfeldt-Jakob disease. *Lancet*, **344**, 1315–1318.
- Arriagada, P.V., Growdon, J.H., Hedley-Whyte, E.T., and Hyman, B.T. (1992). Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer disease. *Neural.*, **42**, 631–639.
- Bellosta, S., Nathan, B. P., Orth, M., Dong, L.-M., Mahley, R.W., and Pitas, R.E. (1995). Stable expression and secretion of apolipoproteins E3 and E4 in mouse neuroblastoma cells produces differential effects on neurite outgrowth. *J. Biol. Chem.*, **270**, 27063–27071.
- Benjamin, R., Leake, A., Edwardson, J.A., McKeith, I.G., Ince, P.G., Perry, R.H., and Morris, C.M. (1994). Apolipoprotein E genes in Lewy body and Parkinson's disease. *Lancet*, **343**, 1565.
- Benjamin, R., Leake, A., McArthur, F.K., Ince, P.G., Candy, J.M., Edwardson, J.A., Morris, C.M., and Bjertness, E. (1994). Protective effect of apoE ϵ 2 in Alzheimer's disease. *Lancet*, **344**, 473.
- Benzing, W.C., and Mufson, E.J. (1995). Apolipoprotein E immunoreactivity within neurofibrillary tangles: relationship to tau and PHF in Alzheimer's disease. *Exp. Neurol.*, **132**, 162–171.
- Bertrand, P., Poirier, J., Oda, T., Finch, C.E., and Pasinetti, G.M. (1995). Association of apolipoprotein E genotype with brain levels of apolipoprotein E and apolipoprotein J (clusterin) in Alzheimer disease. *Mol Brain Res.*, **33**, 174–178.
- Blennow, K., Hesse, C., and Fredman, P. (1994). Cerebrospinal fluid apolipoprotein E is reduced in Alzheimer's disease. *NeuroReport*, **5**, 2534–2536.

- Borghini, I., Barja, E., Pometta, D., and James, R.W. (1995). Characterization of subpopulations of lipoprotein particles isolated from human cerebrospinal fluid. *Biochim. Biophys. Acta*, **1255**, 192–200.
- Boyles, J.K., Notterpek, L.M., and Anderson, L.J. (1990). Accumulation of apolipoproteins in the regenerating and remyelinating mammalian peripheral nerve. *J. Biol. Chem.*, **265**, 17805–17815.
- Boyles, J.K., Pitas, R.E., Wilson, E., Mahley, R.W., and Taylor, J.M. (1985). Apolipoprotein E associated with astrocytic glia of the central nervous system and with nonmyelinating glia of the peripheral nervous system. *J. Clin. Invest.*, **76**, 1501–1513.
- Carlsson, J., Armstrong, V.W., Reiber, H., Felgenhauer, K., and Seidel, D. (1991). Clinical relevance of the quantification of apolipoprotein E in cerebrospinal fluid. *Clin. Chim. Acta*, **196**, 167–176.
- Castano, E.M., Prelli, F., Wisniewski, T., Golabek, A., Kumar, R.A., Soto, C., and Frangione, B. (1995). Fibrillogenesis in Alzheimer's disease of amyloid β peptides and apolipoprotein E. *Biochem. J.*, **306**, 599–604.
- Chartier-Harlin, M.-C., Parfitt, M., Legrain, S., Perez-Tur, J., Brousseau, T., Evans, A., Berr, C., Vidai, O., Roques, P., Gourlet, V., Fruchart, J.-C., Delacourte, A., Rossor, M., and Amouyel, P. (1994). Apolipoprotein E, $\epsilon 4$ allele as a major risk factor for sporadic early and late-onset forms of Alzheimer's disease: analysis of the 19q13.2 chromosomal region. *Hum. Molec. Genet.*, **3**, 569–574.
- Chauhan, A., Pirttila, T., Mehta, P., Chauhan, V.P.S., and Wisniewski, H.M. (1996). Effect of cerebrospinal fluid from normal and Alzheimer's patients with different apolipoprotein E phenotypes on *in vitro* aggregation of amyloid beta-protein. *J. Neurol. Sci.*, **141**, 54–58.
- Christie, R.H., Chung, H., Rebeck, G.W., Strickland, D., and Hyman, B.T. (1996). Expression of the very low density lipoprotein receptor (VLDL-r), an apolipoprotein E receptor, in the central nervous system and in Alzheimer disease. *J. Neuropath. Exp. Neurol.*, **55**, 491–498.
- Christie, R.H., Freeman, M., and Hyman, B.T. (1996). Expression of the macrophage scavenger receptor, a multifunctional lipoprotein receptor, in microglia associated with senile plaques in Alzheimer's disease. *Am. J. Path.*, **148**, 399–403.
- Corder, E.H., Saunders, A.M., Risen, N.J., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Rimmler, J.B., Locke, P.A., Conneally, M., Schmechel, K.E., Small, G.W., Roses, A.D., Haines, J.L., and Pericak-Vance, M.A. (1994). Apolipoprotein E type 2 allele decreases the risk of late onset Alzheimer disease. *Nature Genetics*, **7**, 180–184.
- Cruz, L., Urbanc, B., Buldyrev, S.V., Christie, R., Gomez-Isla, T., Havlin, T., McNamara, M., Stanley, H.E., and Hyman, B.T. (1997). Aggregation and disaggregation of senile plaques in Alzheimer disease. *Proc. Natl. Acad. Sci. USA*, **94**, 7612–7616.
- Czech, C., Moenning, U., Tienari, P., Hartmann, T., Masters, C., Beyreuther, K., and Foerstl, H. (1993). Apolipoprotein E- $\epsilon 4$ allele and Alzheimer's disease. *Lancet*, **342**, 1309–1310.
- Davignon, J., Gregg, R.E., and Sing, C.F. (1988). Apolipoprotein E polymorphism and atherosclerosis. *Arterioscler.*, **8**, 1–21.
- Dawson, P.A., Schechter, N., and Williams, D.L. (1986). Induction of rat E and chicken A-I apolipoproteins and mRNAs during optic nerve degeneration. *J. Biol. Chem.*, **261**, 5681–5684.
- Du, Y., Glinn, M., Dodel, R.C., Bales, K.R., Zhang, Z., Hyslop, P.A., and Paul, S.M. (1997). $\alpha 2$ macroglobulin as a β -amyloid peptide-binding plasma protein. *J. Neurochem.*, **69**, 299–305.
- El Khoury, J., Hickman, S.E., Thomas, C.A., Cao, L., Silverstein, S.C., and Loike, J.D. (1996). Scavenger receptor-mediated adhesion of microglia to β -amyloid fibrils. *Nature*, **382**, 716–719.

- Evans, K.C., Berger, E.P., Cho, C.-G., Weisgraber, K.H., and Lansbury, P.T. (1995). Apolipoprotein E is a kinetic but not a thermodynamic inhibitor of amyloid formation: implications for the pathogenesis and treatment of Alzheimer disease. *Proc. Natl. Acad. Sci. USA*, **92**, 763–767.
- Pagan, A.M., Bu, G., Sun, Y., Daugherty, A., and Holtzman, D.M. (1996). Apolipoprotein E containing high density lipoprotein promotes neurite outgrowth and is a ligand for the low density lipoprotein receptor-related protein. *J. Biol. Chem.*, **271**, 30121–30125.
- Farrer, L.A., Abraham, C.R., Volicer, L., Foley, E.J., Kowall, N.W., McKee, A.C., and Wells, J.M. (1995). Allele $\epsilon 4$ apolipoprotein E shows a dose effect on age at onset of Pick disease. *Exp. Neurol.*, **136**, 162–170.
- Gearing, M., Schneider, J.A., Rebeck, G.W., Hyman, B.T., and Mirra, S.S. (1995). Alzheimer's disease with and without coexisting Parkinson's disease changes: apolipoprotein E genotype and neuropathological correlates. *Neurol.*, **45**, 1985–1990.
- Ghisso, 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.
- Gomez-Isla, T., Hollister, R., West, H., Mui, S., Growdon, J.H., Petersen, R.C., Parisi, J.E., and Hyman, B.T. (1997). Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease. *Ann. Neurol.*, **41**, 17–24.
- Gomez-Isla, T., West, H.L., Rebeck, G.W., Harr, S.D., Growdon, J.H., Locasio, J.J., Perls, T.T., Lipsitz, L.A., and Hyman, B.T. (1996). Clinical and pathological correlates of apolipoprotein E $\epsilon 4$ in Alzheimer disease. *Ann. Neurol.*, **39**, 62–70.
- Goodrum, J.F. (1991). Cholesterol from degenerating nerve myelin becomes associated with lipoproteins containing apolipoprotein E. *J. Neurochem.*, **56**, 2082–2086.
- Greenberg, S.M., Rebeck, G.W., Vonsattel, J.P.G., Gomez-Isla, T., and Hyman, B. (1995). Apolipoprotein E $\epsilon 4$ and cerebral hemorrhage associated with amyloid angiopathy. *Ann. Neurol.*, **38**, 254–259.
- Gregg, R.E., Zech, L.A., Schaefer, E.J., Stark, D., Wilson, D., and Brewer, H.B., Jr (1986). Abnormal *in vivo* metabolism of apolipoprotein E4 in humans. *J. Clin. Invest.*, **78**, 815–821.
- Hahne, S., Norstedt, C., Ahlin, A., and Nyback, H. (1997). Levels of cerebrospinal fluid apolipoprotein E in patients with Alzheimer's disease and healthy controls. *Neurosci. Lett.*, **224**, 99–102.
- Han, S.-H., Einstein, G., Weisgraber, K.H., Strittmatter, W.J., Saunders, A.M., Pericak-Vance, M., Roses, A.D., and Schmechel, D.E. (1994). Apolipoprotein E is localized to the cytoplasm of human cortical neurons: a light and electron microscopic study. *J. Neuropath. Exp. Neurol.*, **53**, 535–544.
- Han, S.-H., Hulette, C., Saunders, A.M., Einstein, G., Pericak-Vance, M., Strittmatter, W.J., Roses, A.D., and Schmechel, A.E. (1994). Apolipoprotein E is present in hippocampal neurons without neurofibrillary tangles in Alzheimer's disease and in age-matched controls. *Exp. Neurol.*, **128**, 13–26.
- Handelmann, G.E., Boyles, J.K., Weisgraber, K.H., Mahley, R.W., and Pitas, R.E. (1992). Effects of apolipoprotein E, β -very low density lipoproteins, and cholesterol on the extensions of neurites by rabbit dorsal root ganglion neurons *in vitro*. *J. Lipid. Res.*, **33**, 1677–1688.
- Harel, A., Fainaru, M., Shafer, Z., Hernandez, M., Cohen, A., and Schwartz, M. (1989). Optic nerve regeneration in adult fish and apolipoprotein A-I. *J. Neurochem.*, **52**, 1218–1228.
- Harr, S.D., Unt, L., Hollister, R., Hyman, B.T., and Mendez, A.J. (1996). Brain expression of apolipoproteins E, J, and AI, in Alzheimer's disease. *J. Neurochem.*, **66**, 2429–2435.

- Harrington, C.R., Louwagie, J., Rossau, R., Vanmechelen, E., Perry, J.H., Perry, E.K., Xuereb, J.H., Roth, M., and Wischik, C.M. (1994). Influence of apolipoprotein E genotype on senile dementia of the Alzheimer and Lewy body types. *Am. J. Pathol.*, **145**, 1472–1484.
- Henderson, A.S., Easteal, S., Jorm, A.F., Mackinnon, A.J., Korten, A.E., Christensen, H., Croft, L., and Jacomb, P.A. (1995). Apolipoprotein E allele ϵ 4, dementia, and cognitive decline in a population sample. *Lancet*, **346**, 1387–90.
- Herz, J. (1993). The LDL-receptor-related protein—portrait of a multifunctional receptor. *Curr. Opin. Lipid*, **4**, 107–113.
- Holtzman, D.M., Pitas, R.E., Kilbridge, J., Nathan, B., Mahley, R.W., Bu, G., and Schwartz, A.L. (1995). Low density lipoprotein receptor-related protein mediates apolipoprotein E-dependent neurite outgrowth in a central nervous system-derived neuronal cell line. *Proc. Natl. Acad. Sci. USA*, **92**, 9480–9484.
- Huang, Y., von Eckardstein, A., Wu, S., Maeda, N., and Assman, G. (1994). A plasma lipoprotein containing only apolipoprotein E and with γ mobility on electrophoresis releases cholesterol from cells. *Proc Natl Acad Sci (USA)*, **91**, 1834–1838.
- Hyman, B.T., Gomez-Isla, T., Briggs, M., Chung, H., Nichols, S., Kohout, F., and Wallace, R. (1996). Apolipoprotein E and cognitive change in an elderly population. *Ann. Neurol.*, **40**, 55–66.
- Hyman, B.T., Marzloff, K., and Arriagada, P.V. (1993). The lack of accumulation of senile plaques or amyloid burden in Alzheimer's disease suggests a dynamic balance between amyloid deposition and resolution. *J. Neuropath. Exp. Neurol.*, **52**, 594–600.
- Hyman, B.T., West, H.L., Rebeck, G.W., Lai, F., and Mann, D.M.A. (1995). Neuropathological changes in Down syndrome hippocampal formation: effect of age and apolipoprotein E genotype. *Arch. Neurol.*, **52**, 373–378.
- 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.
- Ignatius, M.J., Gebicke-Haerter, P.J., Skene, J.H.P., Schilling, J.W., Weisgraber, K.H., Mahley, R.W., and Shooter, E.M. (1986). Expression of apolipoprotein E during nerve degeneration and regeneration. *Proc. Natl. Acad. Sci. USA*, **83**, 1125–1129.
- Ji, Z.-S., Brecht, W.J., Miranda, R.D., Hussain, M.M., Innerarity, T.L., and Mahley, R.W. (1993). Role of heparan sulfate proteoglycans in the binding and uptake of apolipoprotein E-enriched remnant lipoproteins by cultured cells. *J. Biol. Chem.*, **268**, 10160–10167.
- Ji, Z.-S., Fazio, S., Lee, Y.-L., and Mahley, R.W. (1994). Secretion-capture role for apolipoprotein E in remnant lipoprotein metabolism involving cell surface heparan sulfate proteoglycans. *J. Biol. Chem.*, **269**, 2764–2772.
- Jordan, J., Galindo, M.F., Miller, R.J., Reardon, C.A., Getz, G.S. and LaDu, M.J. (1998) Isoforanspecific effect of apolipoprotein E on cell survival and β -amyloid-induced toxicity in rat hippocampal pyramidal neuronal cultures. *J. Neurosci.*, **18**, 195–204.
- Kang, D.E., Saitoh, T., Chen, X., Xia, Y., Masliah, E., Hansen, L.A., Thomas, R.G., Thal, L.J., and Katzamn, R. (1997). Genetic association of the low-density lipoprotein receptor-related protein gene (LRP), an apolipoprotein E receptor, with late-onset Alzheimer's disease. *Neurol.*, **49**, 56–61.
- Kim, D.-H., Iijima, H., Goto, K., Sakai, J., Ishii, H., Kim, H.-J., Suzuki, H., Kondo, H., Saeki, S., and Yamamoto, T. (1996). Human apolipoprotein E receptor 2. *J. Biol. Chem.*, **271**, 8373–8380.
- Knauer, M.F., Orlando, R.A., and Glabe, C.G. (1996). Cell surface APP751 forms complexes with protease nexin 2 ligands and is internalized via the low density lipoprotein receptor-related protein (LRP). *Brain Res.*, **740**, 6–14.

- Koller, W.C., Glatt, S.L., Hubble, J.P., Paolo, A., Troster, A.I., Handler, M.S., Horvat, R.T., Martin, C., Schmidt, K., Karst, A., Wijsman, E.M., Yu, C.-E., and Schellenberg, G.D. (1995). Apolipoprotein E genotypes in Parkinson's disease with and without dementia. *Ann. Neurol.*, **37**, 242–245.
- Koudinov, A.R., Koudinova, N.V., Kumar, A., Beavis, R.C., and Ghiso, J. (1996). Biochemical characterization of Alzheimer's soluble amyloid beta protein in human cerebrospinal fluid: association with high density lipoproteins. *Biochem. Biophys. Res. Commun.*, **223**, 592–597.
- Kounnas, M.Z., Moir, R.D., Rebeck, G.W., Bush, A.I., Argraves, W.S., Tanzi, R.E., Hyman, B.T., and Strickland, D.K. (1995). LDL receptor-related protein, a multifunctional apoE receptor, binds secreted β -amyloid precursor protein and mediates its degradation. *Cell*, **82**, 331–340.
- Krieger, M., and Herz, J. (1994). Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu. Rev. Biochem.*, **63**, 601–37.
- LaDu, M.J., Falduto, M.T., Manelli, A.M., Reardon, C.A., Getz, G.S., and Frail, D.E. (1994). Isoform-specific binding of apolipoprotein E to β -amyloid. *J. Biol. Chem.*, **269**, 23403–23406.
- LaDu, M.J., Pederson, T.M., Frail, D.E., Reardon, C.A., Getz, G., and Falduto, M.T. (1995). Purification of apolipoprotein E attenuates isoform-specific binding to β -amyloid. *J. Biol. Chem.*, **270**, 9039–9042.
- LaDu, M.J., Gilligan, S.M., Lukens, J.R., Cabana, V.G., Reardon, C.A., Van Eldik, L.J. and Holtzman, D.M. (1998) Nascent astrocyte particles differ from lipoproteins in CSF. *J. Neurochem.* (in press).
- Lefranc, D., Vermersch, P., Dallongeville, J., Daems-Monpeurt, C., Petit, H., and Delacourte, A. (1996). Relevance of the quantification of apolipoprotein E in the cerebrospinal fluid in Alzheimer's disease. *Neurosci. Lett.*, **212**, 91–94.
- Ma, J., Yee, A., Brewer Jr, A.Y.H., Das, S., and Potter, H. (1994). amyloid-associated proteins alantichymotrypsin and apolipoprotein E promote assembly of Alzheimer β -protein into filaments. *Nature*, **372**, 92–94.
- Mahley, R.W. (1988). Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science*, **240**, 622–630.
- Mayeux, R., Stern, Y., Ottman, R., Taternichi, 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.
- Merched, A., Blain, H., Visvikis, S., Herbeth, B., Jeandel, C., and Siest, G. (1997). Cerebrospinal fluid apolipoprotein E level is increased in late-onset Alzheimer's disease. *J. Neurol. Sci.*, **145**, 33–39.
- Metzger, R.E., LaDu, M.J., Pan, J.B., Getz, G.S., Frail, D.E., and Falduto, M.T. (1996). Neurons of the human frontal cortex display apolipoprotein E immunoreactivity: implications for Alzheimer's disease. *J. Neuropath. Exp. Neurol.*, **55**, 372–380.
- Minthon, L., Hesse, C., Sjogren, M., Englund, E., Gustafson, L., and Blennow, K. (1997). The apolipoprotein E $\epsilon 4$ allele frequency is normal in fronto-temporal dementia, but correlates with age at onset of disease. *Neurosci. Lett.*, **226**, 65–67.
- Moestrup, S.K., Gliemann, J., and Pallensen, G. (1992). Distribution of the $\alpha 2$ -macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell Tissue Res.*, **269**, 375–382.
- Mori, T., Iijima, N., Kitabatake, K., and Kohsaka, S. (1990). $\alpha 2$ -Macroglobulin is an astroglial-derived neurite-promoting factor for cultured neurons from rat central nervous system. *Brain Res.*, **527**, 55–61.
- Moulard, B., Sefiani, A., Laamri, A., Malafosse, A., and Camu, W. (1996). Apolipoprotein E genotyping in sporadic amyotrophic lateral sclerosis: evidence for a major influence on the clinical presentation and prognosis. *J. Neurol. Sci.*, **139** (Suppl), 34–37.

- Mui, S., Rebeck, G.W., McKenna-Yasek, D., Hyman, B.T., and Brown, R.H. (1995). Apolipoprotein E ϵ 4 allele is not associated with earlier age at onset in amyotrophic lateral sclerosis. *Ann. Neurol.*, **380**, 460–463.
- Nagy, Z.S., Esiri, M.M., Jobst, K.A., Johnston, C., Litchfield, S., Sim, E., and Smith, A.D. (1995). Influence of the apolipoprotein E genotype on amyloid deposition and neurofibrillary tangle formation in Alzheimer's disease. *Neurosci.*, **69**, 757–761.
- Nakagawa, Y., Kitamoto, T., Furukawa, H., Ogomori, K., and Tateishi, J. (1995). Allelic variation of apolipoprotein E in Japanese sporadic Creutzfeldt-Jakob disease patients. *Neurosci. Lett.*, **187**, 209–211.
- 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.*, **541**, 163–166.
- Narita, M., Bu, G., Holtzman, D.M., and Schwartz, A.L. (1997). The low-density lipoprotein receptor-related protein, a multifunctional apolipoprotein E receptor, modulates hippocampal neurite development. *J. Neurochem.*, **68**, 587–595.
- Nathan, B.P., Bellosta, 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.
- Nathan, B.P., Chang, K.-C., Bellosta, S., Brisch, E., Ge, N., Mahley, R.W., and Pitas, R.E. (1995). The inhibitory effect of apolipoprotein E4 on neurite outgrowth is associated with microtubule depolymerization. *J. Biol. Chem.*, **370**, 19791–19799.
- Nicoll, J.A.R., Roberts, G.W., and Graham, D.I. (1995). Apolipoprotein ϵ 4 allele is associated with deposition of amyloid β -protein following head injury. *Nature Med.*, **1**, 135–137.
- Ohm, T.G., Kirca, M., Bohl, J., Schamagl, H., Gross, W., and Marz, W. (1995). Apolipoprotein E polymorphism influences not only cerebral senile plaque load but also Alzheimer-type neurofibrillary tangle formation. *Neurosci.*, **66**, 583–587.
- Ordovas, J.M., Litwack-Klein, L., Wilson, P.W.F., Schaefer, M.M., and Schaefer, E.J. (1987). Apolipoprotein E isoform phenotyping methodology and population frequency with identification of apoE1 and apoE5 isoforms. *J. Lipid. Res.*, **28**, 371–380.
- Paresce, D.M., Ghosh, R.N., and Maxfield, F.R. (1996). Microglial cells internalize aggregates of the Alzheimer's disease amyloid β -protein via a scavenger receptor. *Neuron*, **17**, 553–565.
- Pericak-Vance, M.A., Bebout, J.L., Gaskell, P.C., Yamaoka, L.H., Hung, W.-Y., Alberts, M.J., Walker, A.P., Bartlett, R.J., Haynes, C.A., Welsh, K.A., Earl, N.L., Heyman, A., Clark, C.M., and Roses, A.D. (1991). Linkage studies in familial Alzheimer disease: evidence for chromosome 19 linkage. *Am. J. Hum. Genet.*, **48**, 1034–1050.
- Pickering-Brown, S.M., Mann, D.M.A., Owen, E., Ironside, J.W., de Silva, R., Roberts, D.A., Balderson, D.J., and Cooper, P.N. (1995). Allelic variation in apolipoprotein E and prion protein genotype related to plaque formation and age of onset in sporadic Creutzfeldt-Jakob disease. *Neurosci. Lett.*, **187**, 127–129.
- Pickering-Brown, S.M., Siddons, M., Mann, D.M.A., Owen, F., Neary, D., and Snowden, J.S. (1995). Apolipoprotein E allelic frequencies in patients with lobar atrophy. *Neurosci. Lett.*, **188**, 205–207.
- Pirtilla, T., Lehtimäki, T., Rinne, J., Mattila, K., Frey, H., and Nikkari, T. (1996). The frequency of apolipoprotein E4 allele is not increased in patients with probable vascular dementia. *Acta Neurol Scand*, **93**, 352–354.
- Pitas, R.E., Boyles, J.K., Lee, S.H., Foss, D., and Mahley, R.W. (1987). Astrocytes synthesize apolipoprotein E and metabolize apolipoprotein E-containing lipoproteins. *Biochim. Biophys. Acta*, **917**, 148–161.

- 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. *J. Biol. Chem.*, **262**, 14352–14360.
- Poirier, J., Baccichet, A., Dea, D., and Gauthier, S. (1993). Cholesterol synthesis and lipoprotein reuptake during synaptic remodelling in hippocampus in adult rats. *Neurosci.*, **55**, 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., Hess, M., May, P.C., and E, F.C. (1991). Astrocytic apolipoprotein E mRNA and GFAP mRNA in hippocampus after entorhinal cortex lesioning. *Mol. Brain Res.*, **11**, 97–106.
- Polvikoski, T., Sulkava, R., Haltia, M., Kainulainen, K., Vuorio, A., Verkkoniemi, A., Niinisto, L., Halonen, P., and Kontula, K. (1995). Apolipoprotein E, dementia, and cortical deposition of β amyloid protein. *New Engl. J. Med.*, **333**, 1242–1247.
- Premkumar, D.R., Cohen, D.L., Hedera, P., Friedland, R.P., and Kalaria, R.N. (1996). Apolipoprotein E-epsilon4 alleles in cerebral amyloid angiopathy and cerebrovascular pathology associated with Alzheimer's disease. *Am. J. Path.*, **148**, 2083–2095.
- Puttfarcken, P.S., Manelli, A.M., Falduto, M.T., Getz, G.S., and LaDu, M.J. (1997). Effect of apolipoprotein E on neurite outgrowth and β -amyloid-induced toxicity in developing rat primary hippocampal cultures. *J. Neurochem.*, **68**, 760–769.
- Qiu, W.Q., Borth, W., Ye, Z., Haass, C., Teplow, D.B., and Selkoe, D.J. (1996). Degradation of amyloid β -protein by a serine protease- α 2-macroglobulin complex. *J. Biol. Chem.*, **271**, 8443–8451.
- Rebeck, G.W., Alonzo, N.C., Berezovska, O., Harr, S.D., Knowles, R.B., Growdon, J.H., Hyman, B.T. and Mendez, A.J. (1998) Structure and functions of human cerebrospinal fluid lipoproteins from individuals of different APOE genotypes. *Exp. Neurol.*, **149**, 175–182.
- Rebeck, G.W., Harr, S.D., Strickland, D.K., and Hyman, B.T. (1995). Multiple, diverse senile plaque-associated proteins are ligands of an apolipoprotein E receptor, the α 2-macroglobulin receptor/ low-density-lipoprotein receptor-related protein. *Ann. Neurol.*, **37**, 211–217.
- Rebeck, G.W., Perls, T.T., West, H.L., Sodhi, P., Lipsitz, L.A., and Hyman, B.T. (1994). Reduced apolipoprotein E4 allele frequency in the oldest old Alzheimer's patients and cognitively normal individuals. *Neurol.*, **44**, 1513–1516.
- 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.
- Roesler, N., Wichart, I., and Jellinger, K.A. (1996). Intra vitam lumbar cerebrospinal fluid and serum and postmortem ventricular immunoreactive apolipoprotein E in patients with Alzheimer's disease. *J. Neurol. Neurosurg. Psych.*, **60**, 452–454.
- Roheim, P.S., Carey, M., Forte, T., and Vega, G.L. (1979). Apolipoproteins in human cerebrospinal fluid. *Proc. Natl. Acad. Sci. USA*, **76**, 4646–4649.
- Rubinsztein, D.C., Leggo, J., Chiano, M., Dodge, A., Norbury, G., Rosser, E., and Craufurd, D. (1997). Genotypes at the GluR6 kainate receptor locus are associated with variation in the age of onset of Huntington disease. *Proc. Natl. Acad. Sci. USA*, **94**, 3872–3876.
- 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., and Strittmatter, W.J. (1994). Apolipoprotein E associates with β amyloid peptide of Alzheimer's disease to form novel monofibrils. *J. Clin. Invest.*, **94**, 860–869.
- Saunders, A.M., Schmechel, D., Breitner, J.C.S., and 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., Pericak-Vance, M.A., and Roses, A.D. (1993). Apolipoprotein E ϵ 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., St George-Hyslop, P.H., Pericak-Vance, M.A., Joo, S.H., Rosi, B.L., Gusella, J.F., Crapper-MacLachlan, D.R., Alberts, M.J., Hulette, C., Crain, B., Goldgaber, D., and Roses, A.D. (1993). Association of apolipoprotein E allele ϵ 4 with lateonset familial and sporadic Alzheimer's disease. *Neurol.*, **43**, 1467–1472.
- Schachter, F., Faure-Delanef, L., Guenot, F., Rouger, H., Froguel, P., Leseur-Ginot, L., and Cohen, D. (1994). Genetic associations with human longevity at the ApoE and ACE loci. *Nature Genet.*, **6**, 29–32.
- Schmechel, D.E., Saunders, A.M., Strittmatter, W.J., Crain, B., Hulette, C., 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 Alzheimers disease. *Proc. Natl. Acad. Sci. USA*, **90**, 9649–9653.
- Schwarzman, A.L., Gregori, L., Vitek, M.P., Lyubski, S., Strittmatter, W.J., Enghilde, J.J., Bhasin, R., Silverman, J., Weisgraber, K.H., Coyle, P.K., Zagorski, M.G., Talafoos, J., Eisenberg, M., Saunders, A.M., Roses, A.D., and Goldgaber, D. (1994). Transthyretin sequesters amyloid β protein and prevents amyloid formation. *Proc. Natl. Acad. Sci. USA*, **91**, 8368–8372.
- Segal, M.B. (1993). Extracellular and cerebrospinal fluids. *J. Inher. Metab. Dis.*, **16**, 617–638.
- Shaffer, L.M., Dority, M.D., Gupta-Bansal, R., Frederickson, R.C.A., Younkin, S.G., and Brunden, K.R. (1995). Amyloid β protein (A β) removal by neuroglial cells in culture. *Neurobiol. Aging*, **16**, 737–745.
- Skoog, I., Hesse, C., Fredman, P., Andreasson, L.-A., Palmertz, B., and Blennow, K. (1997). Apolipoprotein E in cerebrospinal fluid in 85-year-old subjects. *Arch. Neurol.*, **54**, 267–272.
- Snipes, G.J., McGuire, C.B., Norden, J.J., and Freeman, J.A. (1986). Nerve injury stimulates the secretion of apolipoprotein E by nonneuronal cells. *Proc. Natl. Acad. Sci. USA*, **83**, 1130–1134.
- Strittmatter, W., and Roses, A. (1996). Apolipoprotein E and Alzheimer's disease. *Ann. Rev. Neurosci.*, **19**, 53–77.
- Strittmatter, W.J., Saunders, A.M., Goedert, M., Weisgraber, K.H., Dong, L.-M., Jakes, R., Huang, D.Y., Pericak-Vance, M., Schmechel, D., and Roses, A.D. (1994). Isoform-specific interactions of apolipoprotein E with microtubule-associated protein tau: implications for Alzheimer disease. *Proc. Natl. Acad. Sci. USA*, **91**, 11183–11186.
- Strittmatter, W.J., Saunders, A.M., Schmechel, D., Pericak-Vance, M., Enghild, J., Salvesen, G.S., and Roses, A.D. (1993). 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., Dong, L.M., Salvesen, G.S., Pericak-Vance, M., Schmechel, D., Saunders, A.M., Goldgaber, D., and Roses, A.D. (1993). Binding of human apolipoprotein E to β A4 peptide: isoform specific effects and implications for late onset Alzheimer disease. *Proc. Natl. Acad. Sci. USA*, **90**, 8098–8102.
- Swanson, L.W., Simmons, D.M., Hofmann, S.L., Goldstein, J.L., and Brown, M.S. (1988). Localization of mRNA for low density lipoprotein receptor and a cholesterol synthetic enzyme in rabbit nervous system by in situ hybridization. *Proc. Natl. Acad. Sci. USA*, **85**, 9821–9825.

- Talbot, C., Lendon, C., Craddock, N., Shears, S., Morris, J.C., and Goate, A. (1994). Protection against Alzheimer's disease with apoE ϵ 2. *Lancet*, **343**, 1432–1433.
- Tooyama, I., Kawamata, T., Akiyama, H., Moestrup, S.K., Gliemann, J., and McGeer, P.L. (1993). Immunohistochemical study of α 2 macroglobulin receptor in Alzheimer and control postmortem human brain. *Mol. Chem. Neuropathol.*, **18**, 153–160.
- Tsai, M.-S., Tangalos, E.G., Petersen, R.C., Smith, G.E., Schaid, D.J., Kokmen, E., Ivnek, R.J., and Thibodeau, N. (1994). Apolipoprotein E: risk factor for Alzheimer's disease. *Am. J. Hum. Genet.*, **54**, 643–649.
- van Gool, W.A., Evenhuis, H.M., and van Duijn, C.M. (1995). A case-control study of apolipoprotein E genotypes in Alzheimer's disease associated with Down's syndrome. *Ann. Neurol.*, **38**, 225–230.
- Weisgraber, K.H. (1990). Apolipoprotein E distribution among human plasma lipoproteins: role of the cysteine-arginine interchange at residue 112. *J. Lipid. Res.*, **31**, 1503–1511.
- Weisgraber, K.H. (1994). Apolipoprotein E: structure-function relationships. *Adv. Prot. Chem.*, **45**, 249–302.
- Weisgraber, K.H., Innerarity, T.L., and Mahley, R.W. (1982). Abnormal lipoprotein receptor-binding activity of the human E apoprotein due to cysteine-arginine interchange at a single site. *J. Biol. Chem.*, **257**, 2518–2521.
- West, H.L., Rebeck, G.W., and Hyman, B.T. (1994). Frequency of the apolipoprotein E ϵ 2 allele is diminished in sporadic Alzheimer disease. *Neurosci. Lett.*, **175**, 46–48.
- Wisniewski, T., and Frangione, B. (1992). Apolipoprotein E: a pathological chaperone protein in patients with cerebral and systemic amyloid. *Neurosci. Lett.*, **135**, 235–238.
- Wolf, B.B., Lopes, M.B.S., VandenBerg, S.R., and Gonias, S.L. (1992). Characterization and immunohistochemical localization of α 2-macroglobulin receptor (low-density lipoprotein receptor-related protein) in human brain. *Am. J. Pathol.*, **141**, 37–42.
- Yan, S.D., Chen, X., Fu, J., Chen, M., Zhu, H., 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.
- Zannis, V.I., and Breslow, J.L. (1981). Human very low density lipoprotein apolipoprotein E isoprotein polymorphism is explained by genetic variation and posttranslational modification. *Biochem*, **20**, 1033–1041.
- 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.
- Zerr, I., Helmhold, M., and Weber, T. (1995). Apolipoprotein E in Creutzfeldt-Jakob disease. *Lancet*, **345**, 68–69.
- Zheng, G., Bachinsky, D.R., Stamenkovic, I., Strickland, D.K., Brown, D., Andres, G., and McCluskey, R.T. (1994). Organ distribution in rats of two members of the low-density lipoprotein receptor gene family, gp330 and LRP/ α 2MR, and the receptor-associated protein (RAP). *J. Histochem. Cytochem.*, **42**, 531–542.

TRANSGENIC MODELS FOR ALZHEIMER'S DISEASE

17.

TRANSGENIC ANIMAL MODELS IN THE DEVELOPMENT OF THERAPEUTIC STRATEGIES FOR ALZHEIMER'S DISEASE

MATTHIAS STAUFENBIEL and BERND SOMMER

Nervous System Research, Novartis Pharma Inc., CH-4002 Basel, Switzerland

INTRODUCTION

Among the major challenges in Alzheimer's disease (AD) research is the generation of authentic animal models which reflect important aspects of the disease pathogenesis. Two primary motives account for the urgent need for such models. They can be excellent research tools when analyzing the molecular mechanisms involved in the development of AD. Without them studies in organisms are largely restricted to *post mortem* material from the brains of AD patients. Furthermore, testing of compounds in a predictive model prior to clinical trials is highly desirable for ethical reasons. Moreover, clinical studies in AD are extremely expensive due to the large groups of patients required as well as their long duration.

CONSIDERATIONS FOR MODEL GENERATION

During recent years much effort went into the generation of transgenic animal models for AD. This approach allows the introduction, deletion or modification of genes implicated in the disease. Known disease-causing genes can be used as starting points for the development of specific models. In this case any relevant phenotype found may be attributed to a biologically relevant cause of AD and the responsible pathogenic mechanism should be similar to the one effective in the disease. Consequently, models generated in this way are likely to enable studies of the causal events leading to AD, although possible species-specific differences still have to be considered. While lesion models, such as those used to test cholinergic drugs, aim at a specific mechanistic hypothesis, these transgenic models are largely independent of mechanistic assumptions and can be used to test the validity of many hypotheses.

Etiologically AD is heterogeneous (St George-Hyslop *et al.* 1990). Yet the clinical phenotype and the neuropathological lesions are not distinctly different among the different forms of AD (Katzman, 1986; Hansen *et al.*, 1988; Ghetti *et al.*, 1992; Mann *et al.*, 1992; Mullan *et al.* 1993 ; Lantos *et al.*,

1994). Furthermore, the mutations in the known AD genes have been shown to lead to common biochemical alterations *in vitro* as well as in affected individuals (Citron *et al.*, 1992 and, 1997; Cai *et al.*, 1993; Suzuki *et al.*, 1994; Tamaoka *et al.*, 1994; Scheuner *et al.*, 1996). Apparently the different causes initiate pathways which at some point merge into a common pathogenic mechanism. Based on these arguments AD can be considered as a single disorder. This notion is essential to the relevance of all transgenic AD models generated to date because the mutated genes used represent rare forms of AD as compared to the much more abundant "sporadic" forms.

AD-linked mutations have been identified in three genes, the β -amyloid precursor protein (APP) gene (Goate *et al.*, 1991; Mullan *et al.*, 1992) and, more recently, in the presenilin (PS1 and PS2) genes (Sherrington *et al.*, 1995; Levy-Lahad *et al.*, 1995; Rogaev *et al.*, 1995). The mutations in all three genes are autosomal dominant with full penetrance and thus appear to be well suited to induce a disease phenotype in transgenic animals. In addition, the ϵ 4 allele of Apolipoprotein E was found associated with an increased risk of AD (Corder *et al.*, 1993). As a susceptibility factor this gene seems less appealing for development of models.

GENERATION OF TRANSGENIC MOUSE LINES OVEREXPRESSING APP

Numerous attempts focusing on transgenic expression of human APP have been made most of which did not lead to an AD-specific phenotype (Quon *et al.* 1991; Lamb *et al.*, 1993; Mucke *et al.*, 1994; Howlands *et al.*, 1995; Andr  *et al.*, 1996; Loring *et al.*, 1996; Malherbe *et al.*, 1996; Czech *et al.*, 1997). One reason for this failure may reside in the amount of mutated APP being insufficient to trigger pathogenesis during the short life span of mice. To obtain high APP expression in the brain we have generated constructs containing a human APP751 cDNA under the control of different neuron-specific promoters (Andr  *et al.*, 1996). In our hands only the Thy-1 promoter gave rise to transgene APP levels matching or exceeding the endogenous mouse APP. During subsequent studies

Table 1. Molecular Characteristics of the APP Transgenic Mice Described*.

<i>Code</i>	<i>Mutation</i>	<i>Expression cassette</i>	<i>hAPP mRNA/ endogenous</i>
APP 14	670/671: Lys/Met→Asn/Leu	human Thy-1/SV40	2
APP 22	670/671: Lys/Met→Asn/Leu 717: Val→Ile	human Thy-1/SV40	2
APP 23	670/671: Lys/Met→Asn/Leu	murine Thy-1	7

*All mice contain an APP751 cDNA.

we used two expression cassettes containing either the human or the mouse Thy-1 promoter together with mutated human APP751 cDNAs (see Table 1). APP 14 mice express APP carrying the "Swedish" double mutation (KM670/671NL; Mullan *et al.*, 1992) under the control of the human Thy-1 promoter, while the same promoter and APP with the combined mutations at positions 670/671 (KM->NL) and 717 (V->I; Goate *et al.*, 1991) is present in APP 22 mice (Andr  *et al.*, 1996; Sturchler-Pierrat *et al.*, 1997). As determined by relative quantitative PCR the mRNA levels exceed endogenous APP mRNA by two-fold in both lines. In the APP 23 line, APP with the "Swedish" double mutation is overexpressed seven-fold, driven by the murine Thy-1 promoter (Sturchler-Pierrat *et al.*, 1997). By *in*

situ hybridization comparable spatial expression patterns are observed in these lines with highest levels of transgene mRNA in the deep cortical layers and the hippocampus (Figure 2A,B). A similar overexpression of APP was found by Western blotting.

DEVELOPMENT OF A β DEPOSITS IN CORTEX AND HIPPOCAMPUS OF APP 22 AND APP 23 TRANSGENIC MICE

Ideally an animal model should reflect both the clinical as well as the pathological alterations associated with AD. A number of studies support the notion that the characteristic pathological lesions start to develop before the typical behavioral alterations become apparent in AD patients (Arriagada *et al.*, 1992; Morris *et al.*, 1996). These behavioral changes of humans obviously are difficult to compare to possible alterations found during behavioral testing of mice. In addition, altered behavioral elements in transgenic mice may be induced nonspecifically due to pleiotropic effects of the transgene. For these reasons we relied on histopathological analysis with emphasis on AD-specific parameters (for reviews see Probst *et al.*, 1991; Selkoe, 1994; Yankner, 1996) in order to identify interesting mouse lines.

In APP 23 mice the first typical A β deposits develop at 6 months of age. They increase dramatically in size and number with increasing age and occupy a substantial area of the cerebral cortex and hippocampus in 24 months old mice (Figure 1B,2D). At this stage deposits are also found in the thalamus, the olfactory nucleus and isolated in the caudate putamen. They appear similar to the ones detected in AD brain (Figure 1A). Almost all A β deposits are stained by Thioflavin S (Figure 1C). The lower expressing APP 22 mouse line shows deposits only after 18 months in the cerebral cortex and the hippocampus (Figure 2C). Although the same quantitative and spatial APP expression is reached in APP 14 mice, no indication of AD pathology could be detected in this mouse line up to an age of two years (Andrä *et al.*, 1996). Apparently the additional mutation in the APP 22 transgene at position 717 (V->I) contributes to the development of pathology. With the higher expression reached in the APP 23 mice the "Swedish" double mutation alone is sufficient to induce A β deposition.

All deposits show immunoreactivity with a number of different A β antibodies which is comparable to the staining obtained on brain sections from AD patients. The A β deposits also react with end-specific antibodies recognizing the amino terminus of A β or its carboxy termini at amino acids 40, 42 and weakly at 43 (Sturchler-Pierrat *et al.*, 1997). It is noteworthy that the immunoreactivity with the A β 42 antibody in APP 22 mice appears similar in intensity to that obtained in sections from the higher-expressing APP 23 mice, reflecting the impact of the mutation at position 717 on the production of the more amyloidogenic A β 1–42 (Cai *et al.*, 1993; Suzuki *et al.*, 1994). Typical plaque-associated proteins in AD such as Heparansulfate proteoglycan (Figure 3H) and Apolipoprotein E which bind to A β are also present in the deposits. During the pathological studies no sex differences were observed with these mice.

In APP 22 mice a few Congo red positive plaques are present in the cortex and the subiculum, however, by silver impregnation most deposits appear as diffuse type (Figure 2E,G). In contrast, the majority of the extracellular deposits in APP 23 mice are intensely stained with Methenamine silver (Figure 2F) indicative of dense core plaques. Already at their first appearance they show Congo red birefringence in polarized light (Figure 2H), a characteristic feature of senile plaques in the brains of

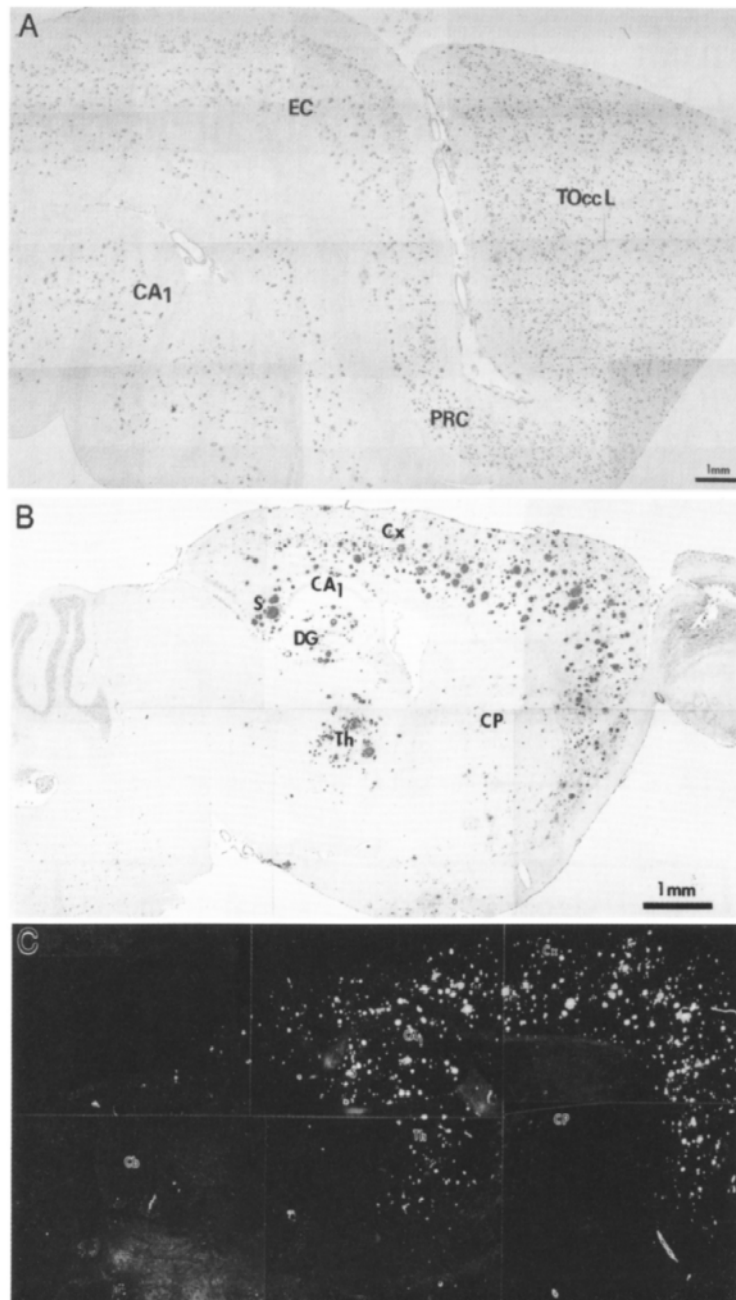


Figure 1. Overview of amyloid plaques in the cerebral cortex of an APP 23 mouse and a human AD brain. Sections through the hippocampal region of a human AD brain (A) and 24 months old APP 23 mouse brains (B, C) were stained with an A β antibody (A, B) or by Thioflavin S (C). CA1, hippocampal CA1 field; Cb, cerebellum; Cp, caudate putamen; Cx, cortex; DG, dentate gyrus; EC, entorhinal cortex; PRC, perirhinal cortex; S, subiculum; Th, thalamus; TOccL, temporal occipital lobe. (See Colour [Plate VIII](#))

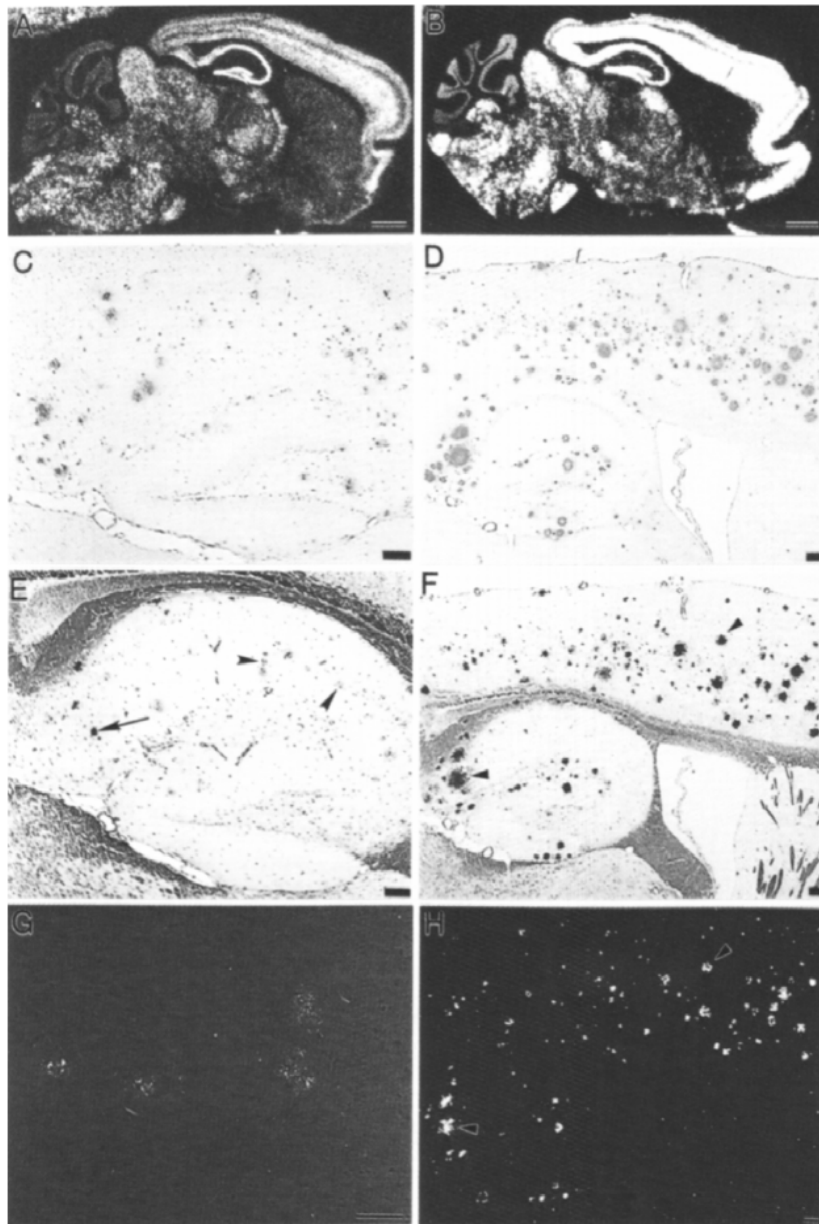


Figure 2. APP mRNA expression and deposition of A β in the brains of transgenic mice. The sagittal sections were obtained from brains of 18 months old APP 22 (A, C, E, G) and 24 months old APP 23 (B, D, F, H) mice. The expression of human APP mRNA is shown by *in situ* hybridization with a specific oligonucleotide probe (A, B; bars correspond to 1 mm). Immunocytochemistry using an antibody specific to A β indicates the distribution of the plaques (C, D). Consecutive sections were stained by Methenamine silver to distinguish compact and diffuse deposits (E, F). Other serial sections were labeled with Congo red to demonstrate the fibrillar nature of the deposits (G, H). A larger magnification is shown in G to visualize the weak Congo red staining. Arrowheads point to identical deposits in F and H. In E the arrow indicates a compact deposit while the arrowheads marks diffuse deposits. Bars correspond to 100 μ m. (See Colour Plate IX)

Alzheimer's disease patients. Direct comparison of the A β or HSPG stainings confirms the more compact nature of the deposits in the APP 23 mice.

INFLAMMATORY PROCESSES IN BRAINS OF APP TRANSGENIC MICE

Inflammatory reactions reminiscent of AD occur in both mouse lines with deposits. Massive astrogliosis and microglia reactivity can be demonstrated in plaque containing brain areas by immunocytochemistry for glial fibrillary acidic protein (Figure 3A) and MAC-1 (Figure 3B). Staining with phosphotyrosine, MHC class II and complement C3 antibodies further suggest an activation of both cell types (Sturchler-Pierrat *et al.*, 1997). Proliferating astrocytes and reactive microglia cells accumulate around the compact deposits but are also found throughout the cerebral cortex and hippocampus. Therefore, a possible increase at diffuse deposits is difficult to detect. This glial response is more pronounced in APP 23 mice correlating with the higher burden of congophilic plaques.

DYSTROPHIC AND DISTORTED NEURITES INCLUDING CHOLINERGIC FIBERS IN THE VICINITY OF PLAQUES

Plaques are surrounded by enlarged dystrophic neurites visualized by neurofilament immunolabeling (Figure 3E) or Holmes-Luxol staining (Staufenbiel *et al.*, 1997). These methods also reveal the distortion of neurites by the deposits leading to a disruption of the normal neuritic pattern in particular in regions with a high plaque load. APP (Figure 3G) and synaptophysin antibodies react with dystrophic structures in the periphery of compact A β deposits very alike the staining of plaques in human AD brain. A reduction of synaptophysin-positive buttons in the neocortex which correlates with the plaque load has been quantified using unbiased stereological methods (Calhoun *et al.*, 1997).

The cholinergic system is strongly implied in the cognitive decline of AD patients. Besides the loss of cholinergic neurons, an accumulation of cholinesterase's in swollen neurites in the vicinity of plaques has been described (Tago *et al.*, 1987; Guelaf *al.*, 1989). Specific staining for acetylcholinesterase in transgenic mouse brains (Figure 3C,D) visualizes a strong labeling of plaque structures resembling swollen, dystrophic neurites. A local distortion of the cholinergic fiber network is seen with this method, too. Preliminary stereological data are in agreement with an effect of A β deposits on cholinergic fibers (Calhoun *et al.*, 1997). This degeneration of the cholinergic system replicates pathological features associated with AD.

PLAQUE LOAD DEPENDENT NEURONAL LOSS IN THE HIPPOCAMPUS

Throughout the neocortex deposition of A β plaques results in a substantial disruption of the normal cytoarchitecture. In the hippocampal pyramidal cell layer, an area of high cell density, in addition a reduction in neuron number adjacent to the A β depositions is apparent (Figure 3F). This reduction does not appear to be caused by passive cell displacement owing to the deposited A β since compression of cell bodies cannot be detected. Instead, the observations suggest a local loss of neurons. A quantitative assessment of these changes by state-of-the-art unbiased stereological methods confirms our

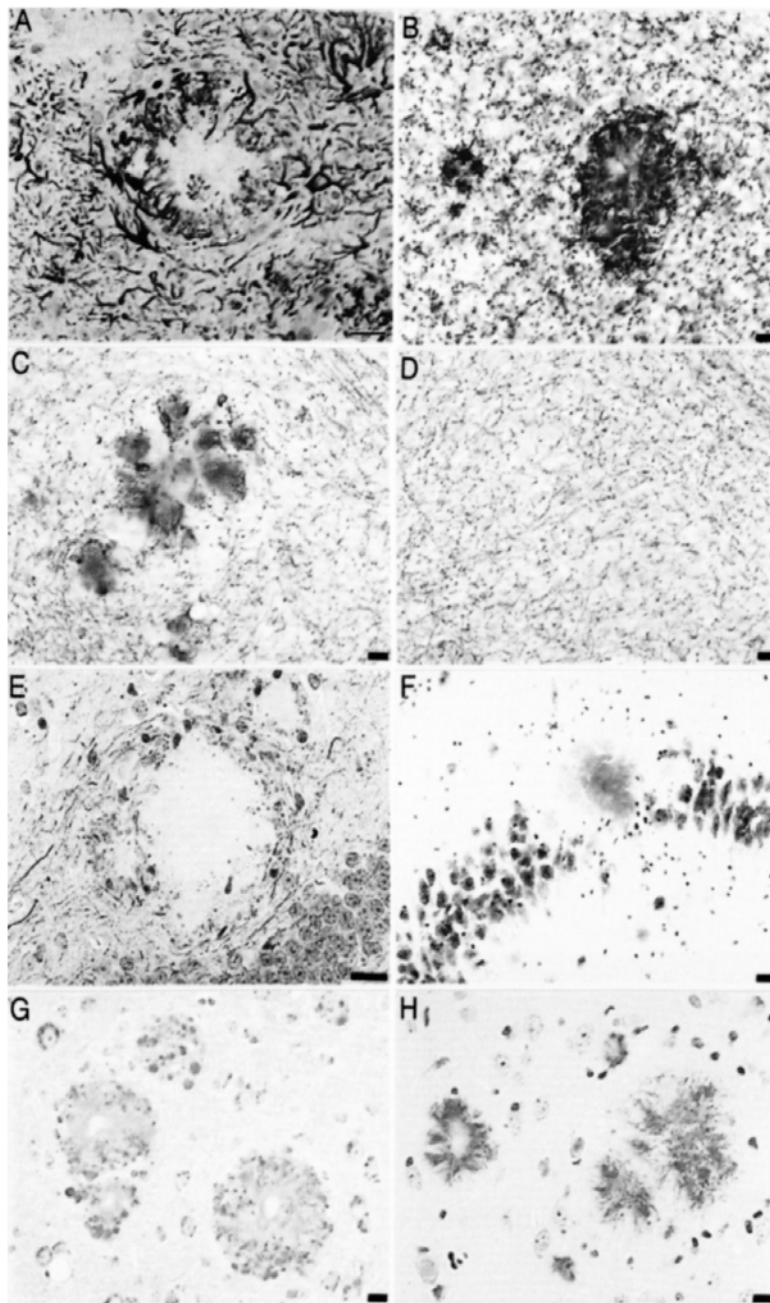


Figure 3. Inflammatory and degenerative processes in the neuropil of 12 months old APP 23 mice. Hypertrophic astrocytes (A) and activated microglia cells (B) are visualized with GFAP or MAC1 antibodies, respectively. Staining for acetylcholinesterase shows a distortion the cholinergic fibers (C) which is not seen in control animals (D) and an association with the plaques. Immunoreaction with neurofilament antibody NF200 reveals dystrophic neurites and neuritic spheroids around the A β deposits (E). The loss of hippocampal pyramidal neurons close to the deposits is shown by toluidine blue staining (F). Typical dystrophic structures probably axonal in origin are detected by labeling with an APP antibody (G). The overexpressed APP can be located in the cell bodies. An antibody to Heparansulphate proteoglycan stains the deposits as expected for this senile plaque associated molecule. Bars correspond to 20 μ m. (See Colour Plate X)

interpretation (Calhoun *et al.*, unpublished data). A significant reduction in neuronal cell bodies is found for the CA1 field of the hippocampus when aged APP 23 mice are compared to controls. This reduction is directly related to plaque load. The neuronal loss is also found when the plaques are located outside the pyramidal cell layer possibly due to an interference with axons or dendrites. In contrast, the neuron number in the neocortex appears to be largely unaffected in the APP 23 mice (Calhoun *et al.*, 1997). With similar assumption-free stereological techniques a selective neuronal loss has been demonstrated in the hippocampal CA1 field of AD patients while no general neuron loss could be detected in the neocortex (West *et al.*, 1994; Regeur *et al.*, 1994).

DISTORTED PROCESSES CONTAINING HYPERPHOSPHORYLATED TAU IN COMPACT PLAQUES

The observation of dystrophic neurites prompted us to search for aspects of neurofibrillary pathology, in particular hyperphosphorylated micro tubule-associated protein tau. Immunoreaction with the AT8 antibody directed against phosphorylated Ser 202/Thr 205 reveals hyperphosphorylated tau in both transgenic mouse lines (Figure 4A-C). The immunostaining is exclusively associated with Congo red positive plaques and highlights curly structures resembling distorted neurites which surround the core of the deposits. Comparable labeling patterns are obtained with the antibody PHF1 directed against tau phosphorylated at Ser 396/402 (Figure 4D). Antibodies R27 and R32 which recognize distinct phosphoepitopes of tau (Ser 396 and Ser 262, respectively) show similar immunoreactivity. Ser 396 represents the abundant proline-directed Ser/Thr-Pro phosphorylation sites in tau while Ser 262, which is located in a microtubule binding domain, is not followed by proline. The staining with the phosphorylation dependent antibodies was specific as it could be blocked with the respective phosphopeptides. We did not see reactivity with antibodies directed against the corresponding non-phosphorylated peptides or with a general tau antibody. Interestingly, staining of similar structures (Figure 4E) is obtained with the Alz-50 antibody which is thought to recognize a conformational change of tau associated with polymerization into filaments (Carmel *et al.*, 1996). This may hint to neurofibrillary pathology in addition to tau hyperphosphorylation. All antibodies do not stain sections from non-transgenic littermate controls. Despite tau hyperphosphorylation and Alz-50 immunoreactivity we failed to detect neurofibrillary tangles or threads by the method of Gallyas (1971) up to now.

Brain extracts from APP 23 mice at 6 months and 15 months of age were also probed for tau hyperphosphorylation using immunoblotting with the AT8 (Figure 4F) and the R27 antibodies. As compared to age-matched littermate controls an elevated phosphorylation of tau can be demonstrated at both time points. In addition, an age-dependent increase in phosphorylation is observed in transgenic mice while no such increase with age is found in the controls. Notwithstanding the elevated phosphorylation, we did not detect a molecular weight shift of tau. When incubated with the phosphorylation independent antibody tau7 no upregulation of total tau protein is seen in transgenic mice (Figure 4G). As judged from the data available to date, the increased tau phosphorylation appears to occur in parallel to A β peptide deposition.

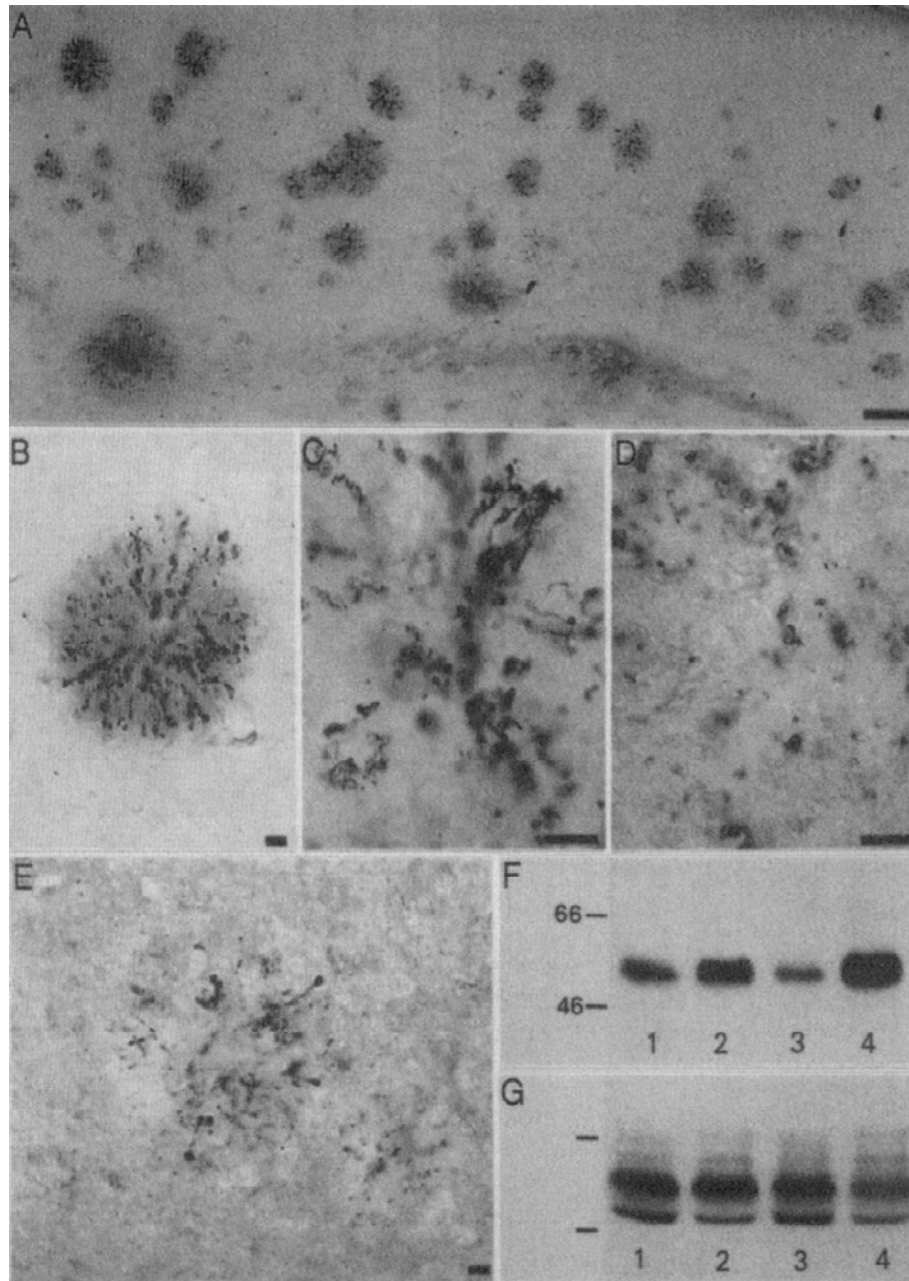


Figure 4. Tau phosphorylation in the brains of 12 months old APP 23 transgenic mice. Free floating sections were reacted with antibodies AT8 (A-C), PHF-1 (D) or Alz-50 (E). Similar structures are stained in Congo red positive plaques which resemble distorted neurites. Bars correspond to 100 μm (A) and 10 μm (B-E). Western blots of brain extracts from APP 23 mice at 6 months (lanes 2) and 15 months (lanes 3) of age and from littermate controls (lanes 1 and 3) were analyzed by immunoblotting using the AT8 antibody (F) or the general tau? antibody (G). Molecular weight markers are given as kDa in F and are indicated by dashes in G. (See Colour Plate XI)

VALIDITY OF THE MODEL AND IMPLICATIONS FOR THE MECHANISM OF AD

Comparison of the various APP transgenic mouse lines we have generated (Andrä *et al.*, 1996; Sturchler-Pierrat *et al.*, 1997) indicates that a considerable overexpression of mutated human APP is one mechanism facilitating the development of AD-like pathology. Overexpression of APP has also been discussed as the cause of AD for Down's syndrome patients (Rumble *et al.*, 1989). Although APP is most likely not mutated in Down's patients, its increased expression alone may be sufficient to trigger AD symptoms considering the longer time required until pathology develops. In addition to the expression level, in transgenic animals a combination of the AD-linked mutations plays an important role in the development of pathology. While both familial AD mutations at 670/671 and 717 in the same transgene of the APP 22 mice trigger pathogenesis, the APP 14 mice which lack the additional V717I mutation but show the same expression level do not develop pathology. These findings support the hypothesis proposing an important contribution of A β 1–42 in the development of AD pathology.

The majority of the A β deposits in APP 22 mice is of the diffuse type with a few congophilic plaques in hippocampus and cortex. APP 23 mice, however, develop almost exclusively congophilic plaques already at their first appearance. These results are difficult to reconcile with a widely presumed hypothesis (Probst *et al.*, 1991) that deposits of the diffuse type are the precursor for congophilic neuritic plaques. Compared to APP 23, the APP 22 mice show a strong A β (42) staining relative to the total A β labeling even though they mostly develop diffuse deposits. Together both mouse lines offer the opportunity to study amyloid plaques for the proposed maturation and to better define the processes and A β species involved.

A pronounced glial reaction as well as markers for inflammatory processes have been found in the brains of AD patients (Mandybur *et al.*, 1990) and in both plaque forming mouse lines. Phosphotyrosine, MAC-1 and MHC class II immunoreactivity is considered indicative of activated microglia in human brain (McGeer *et al.*, 1993) but the precise cellular location of these markers in the mice requires more detailed investigation. Since the C3 component of the complement system can be detected in glia cells surrounding the deposits, it will be of interest to analyze whether this system is fully activated. Despite some indications pointing towards an involvement of inflammation in the pathogenesis of AD (Breitner, 1996) no direct evidence has been presented yet. Blocking inflammatory processes in the APP 23 mice followed by an analysis of their brains for effects on synapse or neuron loss might be an approach to provide such evidence. The mice also appear as a suitable model to investigate the signaling pathways of the inflammatory processes following A β fibril formation and deposition.

Hyperphosphorylation of tau in distorted neurites has been described as an early indicator of neurofibrillary pathology in AD (Braak *et al.*, 1994). The tau pathology observed in the APP transgenic mice appears to resemble this stage. In both AD and transgenic mouse brain the distorted processes containing hyperphosphorylated tau are associated with congophilic but not diffuse plaques. Similarly, induction of tau hyperphosphorylation in neuronal primary cultures has also been reported after treatment with fibrillar but not amorphous A β (Busciglio *et al.*, 1995). Evidence that the tau pathology exceeds increased phosphorylation comes from the reactivity with the Alz-50 antibody. This

antibody was originally described to recognize an AD-specific antigen (Wolozin *et al.*, 1986) and now is considered to react with a discontinuous epitope that is formed following a conformational change of tau associated with polymerization into filaments (Carmel *et al.*, 1996). Therefore neurofibrillary pathology may be expected in these mice, even though they appear to lack neurofibrillary tangles. A detailed ultrastructural study will be necessary to answer this question. It may also help to elucidate the role of tau hyperphosphorylation or aggregation and of neurofibrillary pathology in the loss of synapses and neurons. Irrespective of these open questions, our observations in the brains of transgenic mice expressing APP from two distinct constructs suggest a causal link between A β deposition and tau pathology.

Distorted and dystrophic neurites in the periphery of plaques and a degeneration of the cholinergic system are well described features of AD which are reproduced in the transgenic mice. The reduction of synapse number with increasing plaque load in the neocortex and the decrease in neuron number of the hippocampal CA1 region but not the neocortex parallel observations made in AD brains using similar techniques (West *et al.*, 1994; Regeur *et al.*, 1994). This lack of neuronal loss when the entire neocortex is analyzed does not exclude a loss of neurons in distinct cortical regions, like the entorhinal cortex. The effects found in the hippocampus of transgenic mice are smaller than those observed in *post mortem* AD brain. Among other factors, the shorter time of exposure to amyloid or the increased APP levels suggested to be neuroprotective (Mattson *et al.*, 1993; Bowes *et al.*, 1994; Mucke *et al.*, 1994; Roch *et al.*, 1994) may explain the difference. Nevertheless, these findings provide further evidence for a central role of A β in the pathogenesis of Alzheimer's disease.

Preliminary experiments indicate behavioral alterations in the APP 23 mice. A more complete investigation is currently under way especially to analyze the development of the alterations with aging and to correlate them with the brain pathology. Definite conclusions on learning and memory of these mice will be only possible after completion of these studies.

OTHER TRANSGENIC ANIMAL MODELS

Two other transgenic mouse models have been described to develop age-related cerebral A β deposition characteristic of AD. A four to sixfold overexpression of APP mRNA and a more than 10-fold increase in APP was reported for the PDAPP mice (Games *et al.*, 1995; Rockenstein *et al.*, 1995). Hsiao and colleagues described transgene derived APP levels exceeding endogenous APP by 6-fold (Tg2576; Hsiao *et al.*, 1996). Amyloid plaques develop progressively in both mouse models starting at about 8 months (PDAPP mice; Irizarry *et al.*, 1997a) and 11 to 13 months (Tg2576; Irizarry *et al.*, 1997b) in heterozygous animals. Dense core and diffuse deposits are found but the ratio of both and in particular the fraction of Congo red positive plaques has not been described and may vary among different brain regions (Irizarry *et al.*, 1997a). It is difficult to compare the quantitation of transgene expression in the individual studies, in particular between determinations on the mRNA and protein level. Nevertheless, these observations also support the notion that an overexpression of mutated human APP is a trigger for plaque formation.

Astro- and microgliosis as well as dystrophic neurites were present around the deposits. While a reduction in synaptophysin and MAP2 staining was seen with the PDAPP mice (Games *et al.*, 1995), a decrease of the corresponding mRNAs was not detected (Irizarry *et al.*, 1997a). No neurofibrillary

tangles have been described for both models and the state of tau phosphorylation remains unclear. Although neuropil changes were apparent in both mouse lines, no significant neuronal loss could be found in the hippocampus CA1 or different cortical regions using stereological methods (Irizarry *et al.*, 1997a, b). The reason for this discrepancy with the APP 23 mice is not clear. Differences in the physical state of the amyloid are among the possible explanations. Viewed together, the described plaqueforming mouse models use different promoter constructs, APP isoforms and FAD mutations indicating that plaque formation does not depend on the presence of the APP protease inhibitor domain (Quon *et al.*, 1991), the specific features of a distinct promoter or the APP mutations used. On the other hand, the differences observed in the pathology of the mouse models may not only reflect the expression level or strain differences but may also be due to these intrinsic features of the different promoter constructs used.

Behavioral testing of the Tg2576 mice provided evidence for a reduced spontaneous alternation in a Y maze as well as an impairment in their performance in the Morris water maze (Hsiao *et al.*, 1996). These memory deficits were correlated with A β elevation and plaque formation. However, behavioral deficits in a Y maze were also found in a line transgenic for mouse wild-type APP which did not develop pathology (Tg1874; Hsiao *et al.*, 1995). It has been debated whether the evidence overall is sufficient to demonstrate a memory deficit in the Tg2576 mice (Routtenberg, 1997; Hsiao *et al.*, 1997). Behavioral tests including spontaneous alternation and water maze detected changes in other APP transgenic mice which do not or only in rare cases develop AD-like pathology (Moechars *et al.*, 1996; Quon *et al.*, 1991). On the other hand, all of these mice develop some gliosis indicative of histological changes in response to the transgene. It may be conceivable that behavioral alterations in transgenic animals occur due to possibly subtle, early effects prior to the formation of detectable amyloid deposits. In humans however, amyloid plaques and neurofibrillary tangles were found already in non-demented, apparently presymptomatic elderly (Arriagada *et al.*, 1992; Morris *et al.*, 1996). Transgenic mice expressing an APP C-terminal fragment (C-104) have been reported to show behavioral deficits, impaired LTP and hippocampal neuronal loss (Nalbantoglou *et al.*, 1997). However, due to the absence of robust amyloid plaques or other typical AD pathology and because of the known toxic properties of the C-terminal fragment of APP the relevance of this model to the pathogenesis of AD remains unclear. These examples may illustrate the difficulties in interpreting observed cognition deficits with respect to AD.

Overexpression of the longest human tau isoform in transgenic mice has been shown to lead to its hyperphosphorylation (Götz *et al.*, 1995). While normally axonal, tau was also found in a somatodendritic location but no other AD-related pathology was observed. Specifically, no neurofibrillary tangles could be detected irrespective of the increase in amount and phosphorylation of tau. In rodents tangle formation has never been described and it may not be possible or require additional components even after expression of human tau. At present the role of tau hyperphosphorylation as well as tangle formation in the pathogenic mechanism of AD remains elusive. As a consequence it is currently difficult to decide on the importance of neurofibrillary tangles for the relevance of a murine AD model.

USE FOR DRUG STUDIES

As outlined above dominant mutations have been identified in the APP and the PS1 and PS2 genes which lead to AD. These mutations were shown to affect A β *in vitro* and *in vivo* resulting either in an increase in total A β or in a longer isoform of A β which forms fibrils more readily (Citron *et al.*, 1992 and, 1997; Cai *et al.*, 1993; Suzuki *et al.*, 1994; Tamaoka *et al.*, 1994; Scheuer *et al.*, 1996). *In vitro* A β is toxic to neurons (Yankner, 1996), was described to induce tau hyperphosphorylation (Busciglio *et al.*, 1995) and can activate microglia cells (Meda *et al.*, 1995; Giulian *et al.*, 1996). These findings fit very well to the pathological hallmarks of AD (Probst *et al.*, 1991): the amyloid plaques in which the longer form of A β is preferentially deposited (Roher *et al.*, 1993) and the neurofibrillary tangles containing hyperphosphorylated tau. In addition, gliosis and various inflammatory markers have been described in AD brains and a number of epidemiological studies point to a role of inflammatory processes in the pathogenesis of the diseases (Breitner, 1996). Most efforts to develop causative treatments for AD focus on targets derived from these observations. Depending on the particular approach from which development compounds originate, use of different readouts (endpoints) can be envisioned and the effort for testing may vary considerably. Ultimately, of course, any causal drug treatment should influence more than one pathological parameter. It needs to be mentioned that the following considerations are largely hypothetical since little experience in testing such drugs exists to date.

Compounds inhibiting the generation of A β can initially be analyzed in young mice for their *in vivo* effect on brain A β levels during a short-term study using ELISA or Western blotting as quantitative readout. If the expected reduction of plaque formation shall be demonstrated a much longer treatment will be required to obtain a plaque load sufficient for quantitative assessment. The variability among individual mice which is observed even in inbred strains is an important factor determining the required group size. Depending on these parameters and assuming rapid analyses such studies are estimated to take 12 months in heterozygous animals but could be two to three months faster in homozygous mice. During this time an effect on the described plaque associated alterations (e.g. inflammatory processes, tau hyperphosphorylation) may be analyzed too.

Inhibitors of inflammatory processes, tau phosphorylation or A β fibril formation could be tested in short term studies with older, plaque containing animals for their effect on the respective target mechanisms. Especially drugs of the first two groups should have a relative rapid effect on tau phosphorylation or inflammation. Measuring a hypothetical decrease in plaque formation or growth may require longer test periods. It should be noted that the ability of a compound to interfere with these target mechanisms in transgenic mice may not be a sufficient proof of concept. The deposition of A β , for example, can occur as diffuse or compact plaques which apparently differ in A β structure. In humans only the latter ones are associated with degeneration. Therefore, testing of A β fibril formation inhibitors in an advanced model such as the APP 23 mice should be advantageous over testing in mice which form diffuse deposits (e.g. APP 22 mice). By analysis of downstream events (inflammatory processes, synapse or neuron loss) the APP 23 mice would allow to demonstrate an interference with the appropriate type of fibrils or intermediates. Similar considerations may apply for the other mechanisms, too.

The most difficult testing is foreseen for drugs which prevent A β toxicity *in vitro* or if the aforementioned compound groups are to be tested for their indirect effects on synapse and neuron loss, ultimately the pathologically relevant parameters. Such drug studies could be done in homozygous APP 23 mice during the second year of life. While synapse loss may be quantitated in the neocortex, neuron loss could be analyzed in the CA1 region. However, a study using these parameters will have to rely on time consuming histological assessment with stereological methods as long as no simple histological or biochemical tests have been developed. Possible alternative paradigms, such as the determination of neurotransmitter levels or imaging methods, have not been elaborated in detail until now. Physiological alterations as detected by behavioral or electrophysiological methods are indirect and may be induced nonspecifically. The described (synapto) trophic effects of APP *in vitro* and *in vivo* (Mattson *et al.*, 1993; Bowes *et al.*, 1994; Jin *et al.*, 1994; Mucke *et al.*, 1994; Roch *et al.*, 1994; Small *et al.*, 1994) give a hint as to how undesired physiological properties may be induced by overexpression of APP. At present it may be misleading to rely primarily on such parameters. A detailed characterization and comparison of the different models will first be necessary to avoid possible pitfalls.

Although the APP transgenic mice represent a considerable progress in the development of an authentic model of AD there is clearly room for improvement. While the amyloid plaques and inflammatory processes appear well represented, more extensive neuritic pathology and in particular more and earlier synapse and neuron loss would be desirable. A possible approach to achieve such an improvement is the generation of mice cotransgenic for different AD-linked mutated genes. We are currently testing this possibility by crossbreeding PS1 transgenic mice carrying different mutations into the APP 23 strain.

REFERENCES

- Andrä, K., Abramowski, D., Duke, M., Probst, A., Wiederhold, K.-H., Bürki, 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.
- Arriagada, P.V., Marzloff, B.A., and Hyman, B.T. (1992) Distribution of Alzheimer-type pathologic changes in nondemented elderly individuals matches the pattern in Alzheimer's disease. *Neurology*, **42**, 1681–1688.
- Bowes, M.P., Masliah, E., Otero, D.A.C., Zivin, J.A., and Saitoh, T. (1994) Reduction of neuronal damage by a peptide segment of the amyloid/A4 protein precursor in a rabbit spinal cord ischemia model. *Exp. Neurol.*, **129**, 112–119.
- Braak, E., Braak, H., and Mandelkow E.M. (1994) A sequence of cytoskeletal changes related to the formation of neurofibrillary tangles and neuropil threads. *Acta Neuropathol. Berl.*, **87**, 554–567.
- Breitner, J.C.S. (1996) Inflammatory processes and antiinflammatory drugs in Alzheimer's disease: a current appraisal. *Neurobiol. Aging*, **17**, 789–794.
- Busciglio, J., Lorenzo, A., Yeh, J., and Yankner, B.A. (1995) β -amyloid fibrils induce tau phosphorylation and loss of microtubule binding. *Neuron*, **14**, 879–888.
- Cai, X.-D., Golde, T.E., and Younkin, S.G. (1993) Release of excess amyloid β protein from a mutant amyloid (protein precursor. *Science*, **259**, 514–516.

- Calhoun, M.E., Abramowski, D., Wiederhold, K.-H., Probst, A., Staufenbiel, M., Sommer, B., and Jucker, M. (1997) Impact of amyloid plaques on neuron number, synaptic bouton number, and cholinergic fiber length in a transgenic mouse model of Alzheimer's disease. *Soc. Neurosci. Abst.*, **23**(2), 1637.
- Calhoun, M.E., Abramowski, D., Wiederhold, K.-H., Sturchler-Pierrat, C., Probst, A., Staufenbiel, M., Sommer, B., and Jucker, M. Amyloid plaques and neuronal death in APP transgenic mice. Submitted.
- Carmel, G., Mager, E.M., Binder, L.I., and Kuret, J. (1996) The structural basis of monoclonal antibody Alz50's selectivity for Alzheimer's disease pathology. *J. Biol. Chem.*, **271**, 32789–32795.
- Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A.Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., and Selkoe, D.J. (1992) Mutation of the β -amyloid precursor protein in familial Alzheimer's disease increases β -protein production. *Nature*, **360**, 672–674.
- Citron, M., Westaway, D., Xia, W., Carlson, G., Diehl, T., Levesque, G., Johnson-Wood, K., Lee, M., Seubert, P., Davis, A., Kholodenko, D., Motter, R., Sherrington, R., Perry, B., Yao, H., 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 β -protein in both transfected cells and transgenic mice. *Nature Med.*, **3**, 67–72.
- 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 disease in late onset families. *Science*, **261**, 921–923.
- Czech, C., Delaère, P., Macq, A.F., Reibaud, M., Dreisler, S., Touchet, N., Schombert, B., Mazadier, M., Mercken, L., Theisen, M., Pradier, L., Octave, J.N., Beyreuther, K., and Tremp, G. (1997) Proteolytical processing of mutated human amyloid precursor protein in transgenic mice. *Mol. Brain Res.*, **47**, 108–116.
- Gallyas, F. (1971) Silver staining of Alzheimer's neurofibrillary changes by means of physical development. *Acta Morphol Acad. Sci. Hung.*, **19**, 1–8.
- Games, D., Adams, D., Alessandrini, R., Barbour, R., Berthelette, P., Blackwell, C., Carr, T., Clemens, J., Donaldson, T., Gillespie, F., Guido, T., Hagopian, S., Johnson-Wood, K., Khan, K., Lee, M., Leibowitz, P., Lieberburg, L., 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 β -amyloid precursor protein. *Nature*, **373**, 523–528.
- Geula, C., and Mesulam, M. (1989) Cholinesterases and the pathology of Alzheimer's disease. *Neuroscience*, **33**, 469–481.
- Ghetti, B., Murrell, J., Benson, M.D., and Farlow, M.R. (1992) Spectrum of amyloid β -protein immunoreactivity in hereditary Alzheimer disease with a guanine to thymine missense change at position 1924 of the APP gene. *Brain Res.*, **571**, 133–139.
- Goate, A., Chartier-Harlin, M.-C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., Mant, R., Newton, P., Rooke, K., Roques, P., Talbot, C., PeriakVance, M., Roses, A., Williamson, R., Rossor, M., Qwen, M., and Hardy, J. (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature*, **349**, 704–706.
- Götz, J., Probst, A., Spillantini, M.G., Schäfer, T., Jakes, R., Bürki, K., and Goedert, M. (1995) Somatodendritic localization and hyperphosphorylation of tau protein in transgenic mice expressing the longest human brain tau isoform. *EMBO J.*, **14**, 1304–1313.

- Giulian, D., Haverkamp, L.J., Yu, J.H., Karshin, W., Tom, D., Li, J., Kirkpatrick, J., Kuo, Y.M., and Roher, A.E. (1996) Specific domains of β -amyloid from Alzheimer plaque elicit neuron killing in human microglia. *J. Neurosci.*, **16**, 6021–6037.
- Hansen, L.A., DeTeresa, R., Davies, P., and Terry, R.D. (1988) Neocortical morphometry, lesion counts, and choline acetyltransferase levels in the age spectrum of Alzheimer's disease. *Neurology*, **38**, 48–54.
- Howland, D.S., Savage, M.J., Huntress, F.A., Wallace, R.E., Schwartz, D.A., Loh, T., Melloni, R.H., Degennaro, L.J., Greenberg, B.D., Siman, R., Swanson, M.E., and Scott, R.W. (1995) Mutant and native human β -amyloid precursor proteins in transgenic mouse brain. *Neurobiol. Aging*, **16**, 685–699.
- Hsiao, K., Borchelt, D.R., Olson, K., Johannsdottir, R., Kitt, C., Yunis, W., Xu, S., Eckman, C., Younkin, S., Price, D., Ladecola, C., Clark, H.B., and Carlson, G. (1995) Age-related CNS disorder and early death in transgenic FVB/N mice overexpressing Alzheimer's amyloid precursor proteins. *Neuron*, **15**, 1203–1218.
- 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.
- Hsiao, K., Chapman, P., Nilsen S., Eckman, C., Harigaya, Y., Younkin, S., Yang, E., and Cole, G. (1997) Measuring memory in a mouse model of Alzheimer's disease: Response. *Science*, **277**, 840–841.
- Irizarry, M.C., Soriano, E., McNamara, M., Page, K.J., Schenk, D., Games, D., and Hyman, B.T. (1997a) A β 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) APP_{Sw} transgenic mice develop age-related A β deposits and neuropil abnormalities, but no neuronal loss in CA1. *J. Neuropath. Exptl. Neurol.*, **56**, 965–973.
- Jin, L.W., Ninomiya, H., Roch, J.M., Schubert, D., Masliah, E., Otero, D.A., and Saitoh, T. (1994) Peptides containing the RERMS sequence of amyloid beta/A4 protein precursor bind cell surface and promote neurite extension. *J. Neurosci.*, **14**, 5461–5470.
- Katzman, R.N. (1986) Alzheimer's disease. *N. Engl. J. Med.*, **314**, 964–973.
- Lamb, B.T., Sisodia, S.S., Lawler, A.M., Slunt, H.H., Kitt, C.A., Kearns, W.G., Pearson, P.L., Price, D.L., and Gearhart, J.D. (1993) Introduction and expression of the 400 kilobase precursor amyloid protein gene in transgenic mice. *Nature Genet.*, **5**, 22–30.
- Lantos, P.L., Overstone, I.M.K., Johnson, J., Clelland, C.A., Roques, R., and Rossor, M.N. (1994) Lewy bodies in the brain of two members of a family with the 717 (Val to Ile) mutation of the amyloid precursor protein gene. *Neurosci. Lett.*, **172**, 77–79.
- Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D.M., Oshima, J., Pettingell, W.H., Yu, C.E., Jondro, P.D., Schmidt, S.D., Wang, K., Crowley, A.C., Fu, Y.-H., Guenette, S.Y., Galas, D., Nemens, E., Wijsman, E.M., Bird, T.D., Schellenberg, G.D., and Tanzi, R.E. (1995) Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science*, **269**, 973–977.
- Loring, J.F., Paszty, C., Rose, A., McIntosh, T.K., Mural, H., Pierce, J.E.S., Schramm, S.R., Wymore, K., Lee, V.M.-Y., Trojanowski, J.Q., and Peterson, K.R. (1996) Rational design of an animal model for Alzheimer's disease: introduction of multiple human genomic transgenes to reproduce AD pathology in a rodent. *Neurobiol. Aging*, **17**, 173–182.
- Malherbe, P., Richards, J.G., Bluethmann, M.H., Maggio, J., and Huber, G. (1996) Lack of β amyloidosis in transgenic mice expressing low levels of familial Alzheimer's disease missense mutations. *Neurobiol. Aging*, **17**, 204–214.

- Mann, D.M.A., Jones, D., Snowden, J.S., Neary, D., and Hardy, J. (1992) Pathological changes in the brain of a patient with familial Alzheimer's disease having a missense mutations at codon 717 in the amyloid precursor protein gene. *Neurosci. Lett.*, **137**, 225–228.
- Mattson, M.P., Cheng, B., Culwell, A.R., Esch, E.S., Lieberburg, I., and Rydel, R.E. (1993) Evidence for excitoprotective and intraneuronal calcium-regulating roles for secreted forms of β -amyloid precursor protein. *Neuron*, **10**, 243–254.
- Mandybur, T.L. and Chuirazzi, C.C. (1990) Astrocytes and the plaques of Alzheimer's disease. *Neurology*, **40**, 635–639.
- McGeer, P.L., Kawamata, T., Walker, D.G., Akiyama, H., Tooyama, I., and McGeer, E.G. (1993) Microglia in degenerative neurological disease. *Glia*, **7**, 84–92.
- Meda, L., Cassatella, M.A., Szendrei, G.I., Otvos, L., Baron, P., Villalba, M., Ferrari, D., and Rossi, F. (1995) Activation of microglia cells by β -amyloid protein and interferon- α . *Nature*, **374**, 647–650.
- Moechars, D., Lorent, K., De Strooper, B., Dewachter, L., and Van Leuven, F. (1996) Expression in brain of amyloid precursor protein mutated in the (-secretase site causes disturbed behavior, neuronal degeneration and premature death in transgenic mice. *EMBO J.*, **15**, 1265–1274.
- Morris, J.C., Storandt, M., McKeel, D.W., Rubin, E.H., Price, J.L., Grant, E.A., and Berg, L. (1996) Cerebral amyloid deposition and diffuse plaques in "normal" aging: evidence for presymptomatic and very mild Alzheimer's disease. *Neurology*, **46**, 707–719.
- Mucke, L., Masliah, E., Johnson, W.B., Ruppe, M.D., Alford, M., Rockenstein, E.M., Forss-Petter, S., Pietropaolo, M., Mallory, M., and Abraham, C.R. (1994) Synaptotrophic effects of human amyloid β protein precursors in the cortex of transgenic mice. *Brain Res.*, **666**, 151–167.
- Mullan, M., Crawford, R., Axelman, K., Houlden, H., Lilius, L., Winblad, B., and Lannfelt, L. (1992) A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of β -amyloid. *Nature Genet.*, **1**, 345–347.
- Mullan, M., Tsuji, S., Miki, T., Katasuya, T., Naruse, S., Kaneko, K., Shimizu, T., Kojima, T., Nakano, I., Ogihara, T., Miyatake, T., Overston, L., Crawford, F., Goate, A., Hardy, J., Roques, P., Roberts, G., Luthert, P., Lantos, P., Clark, C., Gaskell, P., Crain, B., and Roses, A. (1993) Clinical comparison of Alzheimer's disease in pedigrees with the codon 717 Val(Ile mutation in the amyloid precursor protein gene. *Neurobiol. Aging*, **14**, 407–419.
- Nalbantoglu, J., Tirado-Santiago, G., Lahsaïni, A., Polrier, J., Goncalves, O., Verge, G., Momoli, F., Weiner, S.A., Massicotte, G., Julien, J.-P., and Shapiro, M.L. (1997) Impaired learning and LTP in mice expressing the carboxy terminus of the Alzheimer amyloid precursor protein. *Nature*, **387**, 500–505.
- Probst, A., Langui, D., and Ulrich, J. (1991) Alzheimer's disease: A description of the structural lesions. *Brain Pathol.*, **1**, 229–239.
- Quon, D., Wang, Y., Catalano, R., Scardina, J.M., Murakami, K., and Cordell, B. (1991) Formation of β -amyloid protein deposits in brains of transgenic mice. *Nature*, **352**, 239–241.
- Regeur, L., Badsberg Jensen, G., Pakkenberg, H., Evans, S.M., and Pakkenberg, B. (1994) No global neocortical nerve cell loss in brains from patients with senile dementia of Alzheimer's type. *Neurobiol. Aging*, **15**, 347–352.
- Roch, J.M., Masliah, E., Roch-Leveq, A.C., Sundsmo, M.P., Otero, D.A., Veinbergs, I., and Saitoh, T. (1994) Increase of synaptic density and memory retention by a peptide representing the trophic domain of the amyloid beta/A4 protein precursor. *Proc. Natl Acad. Sci. USA*, **91**, 7450–7454.
- Rockenstein, E., McConlogue, L., Tan, H., Power, M., Masliah, E., and Mucke, L. (1995) Levels and alternative splicing of amyloid (protein precursor (APP) transcripts in brains of APP transgenic mice and humans with Alzheimer's disease. *J. Biol Chem.*, **47**, 28257–28267.

- Rogaev, E.I., Sherrington, R., Rogaeva, E.A., Levesque, G., Ikeda, M., Liang, Y., Chi, H., Lin, C., Holman, K., Tsuda, T., Mar, L., Sorbi, S., Nacmias, B., Piacentini, S., Amaducci, L., Chumakov, I., Cohen, D., Lannfelt, L., Fraser, P.E., Rommens, J.M., and St George-Hyslop, P.H. (1995) Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. *Nature*, **376**, 775–778.
- Roher, A.E., Lowenson, J.D., Clarke, S., Wolkow, C., Wang, R., Cotter, R.J., Reardon, I.M., ZürcherNeely, H.A., Heinrichson, R.L., Ball, M.J., and Greenberg, B.D. (1993) Structural alterations in the peptide backbone of β -amyloid core protein may account for its deposition and stability in Alzheimer's disease. *J. Biol. Chem.*, **268**, 3072–3083.
- Routtenberg, A. (1997) Measuring memory in a mouse model of Alzheimer's disease. *Science*, **277**, 830–840.
- Rumble, B., Retallack, R., Hilbich, C., Simms, G., Multhaupt, G., Martins, R., Hockey, A., Montgomery, P., Beyreuther, K., and Masters, C. (1989) Amyloid A4 protein and its precursor in Down's syndrome and Alzheimer's disease. *N. Engl. J. Med.*, **320**, 1446–1452.
- Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T.D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-Lehad, E., Vitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wasco, W., Lannfelt, L., Selkoe, D., and Younkin, S. (1996) Secreted amyloid β protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nature Med.*, **8**, 864–870.
- Selkoe, D.J. (1994) Normal and abnormal biology of the β -amyloid precursor protein. *Annu. Rev. Cell Biol.*, **10**, 373–403.
- Sherrington, R., Rogaev, E.I., Liang, Y., Rogaeva, E.A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., Tsuda, T., Mar, L., Foncin, J.-E., Bruni, A.C., Montesi, M.P., Sorbi, S., Rainero, L., Pinessi, L., Nee, L., Chumakov, I., Pollen, D., Brookes, A., Sanseau, P., and St George-Hyslop, P.H. (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature*, **375**, 754–760.
- Small, D.H., Nurcombe, V., Reed, G., Clarris, H., Moir, R., Beyreuther, K., and Masters, C.L. (1994) A heparin-binding domain in the amyloid protein precursor of Alzheimer's disease is involved in the regulation of neurite outgrowth. *J. Neurosci.*, **14**, 2117–2127.
- Staufenbiel, M., Sturchler-Pierrat, C., Abramowski, D., Duke, M., Wiederhold, K.-H., Mistl, C., Rothacher, S., Ledermann, B., Bürki, K., Frey, P., Paganetti, P.A., Waridel, C., Calhoun, M.E., Jucker, M., Probst, A., and Sommer, B. (1997) Pathological features in APP transgenic mice resembling those of Alzheimer's disease. In: E. Friedmann and L.M. Savage (eds.), *Alzheimer's disease II: Exploiting mechanisms for drug development and diagnosis*, IBC Library Series, International Business Communications, Southborough, MA, USA, pp. 4.2.1–4.2.12.
- St George-Hyslop, P., Haines, J.L., Farrer, L.A., Polinsky, R., Van Broeckhoven, C., Goate, A., Crapper McLachlan D.R., Orr, H., Bruni, A.C., Sorbi, S., Rainero, I., Focin, J.-E., Pollen, D., Catu, J.-M., Tupler, R., Voskresenskaya, N., Mayeux, R., Growdon, J., Fried, V.A., Myers, R.H., Nee, L., Backovens, H., Martin, J.-J., Rossor, M., Owen, M.J., Mullan, M., Percy, M.E., Karlinsky, H., Rich, S., Heston, L., Montesi, M., Mortilla, M., Nacmias, N., Gusella, J., Hardy, J.A., and other members of the FAD collaborative study group. (1990) Genetic linkage studies suggest that Alzheimer's disease is not a single homogeneous disorder. *Nature*, **347**, 194–197.
- Sturchler-Pierrat, C., Abramowski, D., Duke, M., Wiederhold, K.-H., Mistl, C., Rothacher, S., Ledermann, B., Bürki, K., Frey, P., Paganetti, P.A., Waridel, C., Calhoun, M.E., Jucker, M., Probst, A., Staufenbiel, M., and Sommer, B. Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 13287–13292.

- Suzuki, N., Cheung, T.T., Cai, X.-D., Odaka, A., Otvos, L., Eckman, C., Golde, I.E., and Younkin, S.G. (1994) An increased percentage of long amyloid β protein secreted by familial amyloid β protein precursor (β APP717) mutants. *Science*, **264**, 1336–1340.
- Tago, H., McGeer, P.L., and McGeer, E.G. (1987) Acetylcholinesterase fibers and the development of senile plaques. *Brain Res.*, **406**, 363–369.
- Tamaoka, A., Odaka, A., Ishibashi, Y., Usami, M., Sahara, N., Suzuki, N., Nukina, N., Mizusawa, H., Shoji, S., Kanazawa, and I. Mori, H. (1994) APP717 missense mutation affects the ratio of amyloid β protein species ($A\beta$ 1–42/43 and $A\beta$ 1–40) in familial Alzheimer's disease brain. *J. Biol. Chem.*, **269**, 32721–32724.
- West, M.J., Coleman, P.D., Flood, D.G., and Troncoso, J.C. (1994) Differences in the pattern of hippocampal neuron loss in normal ageing and Alzheimer's disease. *Lancet*, **344**, 769–772.
- Wblozin, B.L., Pruchnicki, A., Dickson, D.W., and Davies, P. (1986) A neuronal antigen in the brains of Alzheimer's disease patients. *Science*, **232**, 648–650.
- Yankner, B.A. (1996) Mechanisms of neuronal degeneration in Alzheimer's disease. *Neuron*, **16**, 921–932.

18.

PRESENILINS IN TRANSGENIC MICE

KAREN DUFF

Mayo Clinic, Jacksonville

Transgenic modeling of a disease such as AD aims to generate an animal that recreates the human phenotype both for scientific investigation, and as a model system in which to test therapeutic agents designed to alleviate, or inhibit the disease in humans. Although it is proving difficult to recreate the exact disease in mice, some features of the phenotype have been successfully modeled. This has allowed us to explore both the biology and the mechanisms involved in the pathogenesis of the disease.

APP TRANSGENIC MODELS

Transgenic approaches to modeling AD began in 1991 with the discovery of the first pathogenic mutations in APP (Goate *et al.*, 1991). Early attempts to model the disease were considered by many to have failed as none showed the pathognomic features of the disease, which include beta amyloid (A β) containing plaques and tau containing tangles. Since 1995, transgenic modeling has regained credibility following the publication of the PDAPP mouse in 1995 (Games *et al.*, 1995) and the Tg2576 mouse in 1996 (Hsiao *et al.*, 1996). Both these mice display A β containing deposits in regions of the brain affected by AD. The success of these mice is almost certainly related to the very high levels of A β generated through the use of cleverly designed transgenes as it appears that elevation of A β levels above a critical concentration will initiate the fibrillarization process which leads to extracellular deposition of both A β 1–42 and A β 1–4. Although these mice are excellent model systems in which to study A β elevation and deposition, they are not complete models of Alzheimer's disease as neither show tau containing tangles or overt neuronal loss.

PRESENILIN TRANSGENIC MODELS

The publication of the first AD causing mutations in presenilin-1 (PS1) (Sherrington *et al.*, 1995) led to the rapid generation of three different transgenic mouse lines overexpressing PS1 cDNAs. These mice were made almost simultaneously by groups at the University of South Florida (Duff *et al.*, 1996), Johns Hopkins (Borchelt *et al.*, 1996) and the University of Toronto (Citron *et al.*, 1997). The mice

differed in their transgene design (type of promoter, mutation) and in the strain of mice used but were similar in achieving high levels of protein production (1–3 fold over endogenous) in neuronal regions of the brain. The mice demonstrated several properties of presenilin biology. Full length PS1 (at 46 kDa) was only seen in mice producing large amounts of human protein as expected from studies of transfected cells that show that the protein is rapidly turned over. The holoprotein undergoes endoproteolytic cleavage in a highly regulated manner, generating terminal derivatives that accumulate to saturable levels at a 1:1 stoichiometry (Thinakaram *et al.*, 1996). This was also seen in the mouse brain where the transgene derived protein was processed correctly into 18 kDa N terminal and 28 kDa C terminal fragments. The human fragments have slightly retarded mobility on a gel compared to the endogenous mouse PS1 derivatives and when the levels of both mouse and human derivatives were directly compared, it was found that the presence of the human protein led to diminished accumulation of the mouse PS1 fragments (Lee *et al.*, 1997). Both transgenic mice and transfected cells overexpressing an exon 9 deletion (Δ E9) cDNA do not show cleavage of the mutant protein but, interestingly, the mice still show a reduction in the amount of mouse PS1 fragments (Lee *et al.*, 1997). The reason for this diminution in the mouse cleavage products is as yet unclear. Perhaps the most interesting observation to have come from the mice so far is that the fragments derived from mutant PS1 accumulate to a greater degree than fragments from wild type PS1 (Lee *et al.*, 1997). Moreover, the uncleaved Δ E9 mutant cDNA also accumulates to a greater degree than the fragments derived from wild type human PS1 (Lee *et al.*, 1997). These observations together suggest that the mutations in PS1 somehow affect the protein's metabolism but how that relates to the pathogenesis of AD remains to be seen.

Perhaps the greatest insight into how mutations in PS1 might cause Alzheimer's disease has come from the analysis of A β levels in mice overexpressing mutant and wild type PS1 transgenes. A report by Scheuner *et al.*, (1996) on A β levels in fibroblasts from AD patients with PS1 mutations first indicated a link between PS1 and APP metabolism. Reports have now been published that describe the same effect in mouse brain tissue in all three sets of PS1 transgenic mice. The first report (Duff *et al.*, 1996) examined the levels of mouse A β 1–40 and 1–42(43) using a sandwich Elisa system. The two subsequent reports (Borchelt *et al.*, 1996 and Citron *et al.*, 1996) examined the level of human A β derived from a mutant human APP transgene that had been crossed into the PS1 mice. All three reports showed essentially the same results: overexpressing mutant PS1 in the brains of transgenic mice led to the elevation of A β 1–42, but not A β 1–40. This effect was a direct result of the mutation and not overexpression of the human protein as the overexpression of wild-type PS1 did not have any significant effect on A β levels. The fact that AD causing mutations in APP and PS1 both lead to an elevation in A β strongly suggests that this event is a significant and possibly crucial mechanism underlying the etiology of Alzheimer's disease. So far, the elevation of A β 1–42 is the only AD related phenotype reported for the mutant PS1 transgenic mice. Mice have been examined for AD related changes in histopathology at 1 year of age, but no obvious changes have been observed. The mice are currently undergoing intensive analysis in several labs for any changes in behavior, electrophysiology, pathology and biochemistry but the results are likely to be subtle, especially if the AD phenotype in PS1 linked cases is related solely to A β levels and not to a defect in the PS1 protein itself.

Transgenic Crosses

One of the great advantages of transgenic animals is that different mice can be mated together so that the effect of transgene interaction on the disease phenotype can be observed. This type of cross has been performed between the mutant APP transgenic line, Tg2576 (Hsiao *et al.*, 1996) and a mutant PS1 line, PDPS1 (Duff *et al.*, 1996). The Tg2576 line has been shown to develop Ab deposits in the brain between 9 and 12 months of age, and this event is temporally linked to Ab elevation (Hsiao *et al.*, 1996). The PDPS1 line shows an approximately 2 fold elevation of Ab1–42(43) from birth (Duff *et al.*, 1996). When these two animals are mated together, Ab containing deposits are observed in the cortex and hippocampus as early as 12 weeks of age (Holcomb *et al.*, 1998; Duff *et al.*, unpublished). It would appear therefore, that an approximate doubling in Ab1–42 levels, evoked by the PS1 mutant transgene, can accelerate the deposition process by several months. Because the doubly transgenic animals routinely form deposits at approximately 12 weeks of age, they will be a useful resource for the study of drugs designed to either reduce Ab levels or break down deposits. In addition, as the mice have a severe Ab pathology for an extended time relative to singly transgenic APP mice, other features of the disease (such as tau abnormalities or major cell loss) may become apparent as the animals age. It may, however, be necessary to cross them with yet more transgenic animals such as human tau expressing mice to try and generate a more complete phenotype.

To date, there are no published reports of PS2 mutant mice, but the effect of PS2 transgene expression on A β levels is expected to be the same in mice as has been reported for transfected cells (Tomita *et al.*, 1997). There are also no reports of targeted (knockin) mutant PS 1/2 mice although they are currently under construction in several labs. Although each type of transgenic mouse may not recreate the expected disease phenotype, the potential to cross different animals together to study both the biochemical and physiological consequence of gene interaction makes the continued generation of transgenic animals carrying Alzheimer's associated genes a valid pursuit.

REFERENCES

- 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 1–42/1–40 Ratio *in vitro* and *in vivo*. *Neuron*, **17**, 1005–1013.
- Citron, M., Westaway, D., Xia, W., Carlson, G., Diehl, T., Levesque, G., Johnson-Wood K., Lee, M., Seubert, P., Davis, A., Kholodenko, D., Motter, R., Sherrington, R., Perry, B., Yao, H., Strome, R., Lieberburg, I., Rommens, J., Kim, S., Schenk, D., Fraser, P., St George Hyslop, P., and Selkoe, D.J. (1997). Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid β -protein in both transfected cells and transgenic mice. *Nature Medicine*, **3**, 67–72.
- Duff, K., Eckman, C., Zehr, C, Yu, X., Prada, C-M., Perez-tur, J., Hutton, M., Buee, L., Harigaya, Y, Yager, D., Morgan, D., Gordon, M.N., Holcomb, L., Refolo, L., Zenk, B., Hardy, J., and Younkin, S. (1996) Increased amyloid- β 42 (43) in brains of mice expressing mutant presenilin 1. *Nature*, **383**, 710–713.
- Games, D., Adams, D., Alessandrini, R., Barbour, R., Berthelette, P., Blackwell, C., Carr, T., Clemena, J., Donaldson, T., Gillespie, F., Guido, T., Hagopian, S., Johnson-Wood, K., Khan, K., Lee, M., Leibowitz, P., Leiberberg, I., Little,

- S., Masilah, 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., Woloizin, B., and Zhao, J., (1995) Alzheimer type neuropathology in transgenic mice overexpressing V717F β -amyloid precursor protein. *Nature*, **373**, 523–527.
- Goate, A.M., Chartier-Harlin, M.C., Mullan, M.C., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., living, N., James, L., Mant, R., Newton, P., Rooke, K., Roques, P., Talbot, C, Pericak-Vance, M., Roses, A., Williamson, R., Rossor, M.N., Owen, and Hardy, J. (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature*, **349**, 704–706.
- Holcomb, L., Gordon, M.N., McGowan, E., Yu, X., Benkovic, S., Jantzen, P., Wright, K., Saad, I., Mueller, R., Morgan, D., Sanders, S., 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. *Nature Medicine*, **1**, 97–100.
- Hsiao, K., Chapman, P., Nilsen, S., Eckman, C., Harigaya, Y., Younkin, S., Yang, F., and Cole, G. (1996) Correlative memory deficits, $A\beta$ elevation and amyloid plaques in transgenic mice. *Science*, **274**, 99–102.
- Lee, M.K., Borchelt, D.R., Kim, G., Thinakaran, G., Slunt, H.H., Ratovitski, T., Martin, L.J., Kittur, A., Gandy, S., Levey, A.I., Jenkins, N., Copeland, N., Price, D.L., and Sisodia, S.S. (1997) Hyperaccumulation of FAD-linked presenilin 1 variants *in vivo*. *Nature Medicine*, **3**, 756–760.
- Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T.D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-Lahad, E., Viitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wasco, W., Lannfelt, L., Selkoe, D., and Younkin, S. (1996) Secreted amyloid bprotein similar to that in the senile plaques of Alzheimer's disease is increased *in vivo* by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nature Medicine*, **2**(8), 864–870.
- Sherrington, R., Rogaev, E.I., Liang, Y., Rogaeva, E.A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Homan, K., Tsuda, T., Mar, L., Foncin, J.F., Bruni, A.C., Montesi, M.P., Sorbi, S., Rainero, I., Pinessi, L., Nee, L., Chumakov, I., Pollen, D., Brookes, A., Sanseau, P., Polinsky, R.J., Wasco, W., Da Silva H.A.R., Haines, J.L., Pericak-Vance, M.A., Tanzi, R.E., Roses, A.D., Fraser, P.E., Rommens, J.M., and St. George Hyslop, P.H. (1995) Cloning of a gene bearing missense mutations in early onset familial Alzheimer's disease. *Nature*, **375**, 754–760.
- Thinakaran, G., Borchelt, D.R., Lee, M.K., Slunt, H.H., Spitzer, L., Kim, G., Ratovitsky, T., Davenport, F., Nordstedt, C., Seeger, M., Hardy, J., Levey, A.I., Gandy, S.E., Jenkins, N.A., Copeland, N.G., Price, D.L., and Sisodia, S. (1996) Endoproteolysis of presenilin-1 and accumulation of processed derivatives *in vivo*. *Neuron*, **17**, 181–190.
- Tomita, T., Muruyama, K., Saido, T.C., Kume, H., Shinozaki, K., Tokuhira, S., Capell, A., Walter, J., Gruenberg, J., Haass, C., Iwatsubo, T., and Obata, K. (1997) The presenilin 2 mutation (N141I) linked to familial Alzheimer disease (Volga German families) increases the secretion of amyloid beta protein ending at the 42nd (or 43rd) residue. *Proc. Natl. Acad. Sci. USA*, **94**(5), 2025–2030.

INDEX

A

α 2-macroglobulin 296–299, 303, 305

α -synuclein 123, 124, 130, 131

acetylcholinesterase 314, 315

actin filaments 23–24, 42

age-of-onset 52, 53, 59–61, 66

aggregation assay 30

Alg-3 255–256, 257, 261, 263, 276

allosteric interaction 82

aluminium 30, 31, 36, 39, 41, 42

Alz-50 316, 319

Alzheimer susceptibility locus (AD3) 54

Amyloid β -peptide/protein (A β) 85, 95, 98, 100, 141, 153,

163, 200, 212, 231, 247, 261, 319

A β _{1–40} 103, 153

A β _{1–42} 103, 153

clearance 296

deposition 154, 159, 294, 311

dimeric 183, 184

Dutch peptide 185

extracellular concentration 154, 155, 159

fibril formation 321

intracellular A β 117, 141, 157, 159

insoluble A β 154

oligomers 178, 181, 184

oligomerization 177

protofibril 178

secreted A β 117, 153

soluble dimer 182

toxicity 322

vascular A β 154

Amyloid precursor protein (APP) 75, 80, 81, 84, 95, 133, 153, 163, 201, 212, 230, 231, 236, 277, 283, 298

anticoagulant activity of APP 85

Cu(I) complex 80

endogenous 240

expression pattern 111

Flemish mutation 185

intramembrane cleavage 243

isoforms 111

KPI 83, 84

mutations 114, 156, 310

proteolytic processing 154, 240

soluble APP (APPs) 97

soluble APP alpha (sAPPa) 86

soluble APP beta (sAPPb) 86

Swedish mutation 100, 136, 155

transgenic mice 87

amyloid angiopathy 3–7, 10, 15

amyloid plaques 140–142, 163, 235

compact (burned-out) 7, 321

component 102

dense core 311

diffuse 5, 153, 321

neuritic 153

primitive 5

- senile (classical) 4, 111, 163
- animal models 309
- antisense 23
- apoA-I 293
- apolipoprotein E (apo E) 3, 11, 14, 15, 30, 52, 291, 298, 311
- apolipoprotein J (apo J) 3
- apical transport 144
- APLP1 75, 81
- APLP2 75, 81, 85, 87, 91
 - APLP2-KPI domain 85
- apoptosis 142, 197, 211, 247, 249, 251, 256–258, 260, 264, 267–269, 283
 - age-related 157
- appican 133, 148, 149
- APPL 81
- aprotinin 178–180
- astrocytes 5–8, 12, 141, 292, 314
- AT8 316, 317
- autosomal dominant forms of AD 49
- axonal transport 143–145, 147
- axons 138, 143, 145

- B
- β A4-peptide see Amyloid β -peptide ($A\beta$)
- β -Amyloid see Amyloid β -peptide ($A\beta$)
- β -Amyloid precursor protein (β APP)
 - see amyloid precursor protein (APP)
- Bcl-2 248, 249, 250, 260, 271
- Bcl-X 249, 260
- β -protein precursor (β PP) see Amyloid precursor protein
- basement membrane 10
- basolateral transport 144
- brefeldin A (BFA) 116

- C
- CADASIL 229
- Caenorhabditis elegans (*C. elegans*) 207, 219–231, 256
- calcium 250, 263, 269
- calpain inhibitor I 121
- Campanot chambers 143
- carbachol 280
- carboxyl terminal fragments 239
- casein kinase (CK) 278
 - CK-1 278
 - CK-2 278
- caspase 197, 247, 250, 257, 258, 270
 - caspase 3 211, 250
- cathepsin D 141
- caudate putamen 311
- Ced-3 249, 250
- Ced-4 249, 251
- Ced-9 249
- cell death 199
- cell-free models 29
- cell matrix interaction 75
- cholesterol 293
- cholinergic system 314, 319
- chromosome 1 57, 154, 207
- chromosome 14 AD3 54, 154
- chromosome 19 52
- circular dichroism spectroscopy (CD) 167
- classic complement cascade 156
- coagulation cascade 83
- coagulation factor 84
- codon 717 102
- co-immunoprecipitation 196, 213
- collagen 82
- complement system 318
- conformational change 102, 316
- Congo Red 167, 311
- conophilic amyloid angiopathy 174
- Copper 77
 - Copper binding motif 78
 - Copper-dependent turnover 79
 - Copper reductase 79
 - Copper transport 78
 - Copper uptake 78
 - Copper/Zinc SOD (Cu/Zn-SOD) 80
- cortex 311
- cps-3 223, 224
- critical concentration c^* 170, 175
- crosslink 196, 213

D

D-aspartate 27
 death adapter 251, 258
 degeneration 322
 Delta 229, 230
 dementia pugilistica 11
 dendritic transport 144
 diffuse Lewy body disease 11
 diffusion coefficient D 167
 DNA fragmentation 256, 265, 267
 Down's syndrome 11, 50, 113, 318
 Drosophila 207, 220, 221, 225
 drug studies 321

E

early-onset dominant pedigrees 60
 early onset familial AD (FAD) 154
 electron microscopy 181
 ELISA see sandwich ELISA
 elongation 165, 187
 elongation rate 173, 175
 embryonic stem cells 236
 endocytosis 135
 endoplasmic reticulum (ER) 116, 117, 137, 210, 235
 endosome/lysosome 99, 115
 entorhinal cortex 27
 exon trapping 48

F

familial AD 61, 197
 fibers 163, 181, 183
 fiber-fiber association 174
 fibrillogenesis 164, 184, 187, 296
 fibril elongation 170
 fibril formation 165
 fibril length 170, 175
 fibril nucleation 173
 fibrillogenesis inhibitors 172
 fibrillogenesis intermediates 177
 fibroblasts 154–156
 fibronectin 31
 formic acid 154

free radicals 248, 268, 269

G

gain of function 57
 gelation 165
 genetic epidemiology 47
 genetic risk factors 291
 gene tracking 48
 genomic structure 55
 glycation 25
 glycosaminoglycans 29
 Golgi 115, 210
 GSK-3 β 30

H

hemostasis 82, 85
 heparin 19, 29, 35, 43
 heparin sulfate proteoglycans 293, 311
 hereditary cerebral hemorrhage with amyloidosis-Dutch
 type (HCHWA-D) 11, 50, 173, 184
 high density lipoproteins 292
 high molecular weight kininogen (HMK) 84
 hippocampus 311
 hippocampal neurons 138
 histones 252
 horse radish peroxidase 139
 Huntington's disease 123
 hydrodynamic radius R_H 167
 hyperphosphorylation 316

I

immunocytochemistry 195, 210
 inflammatory processes/inflammation 314, 318
 inherited susceptibility 47
 in situ hybridization 210, 310
 interleukin converting enzyme (ICE) 250
 intermediate compartment 116

J

June Kinase 247, 258, 260–271
 JNK cascade 258, 259, 261

K

kinetics 165, 170, 172, 174, 175, 177, 184–187
Kunitz-type inhibitor 83, 298

L

lactacystin 122, 125
lag-2 227, 229, 230, 234
laminin 31, 33, 40
late onset multiplex pedigrees 60
Lewy Body 123
Lin-12 199, 226–231
 mutant 228, 229
 multivulva 226, 227
 signaling 226–229
 suppressor 227
 vulvaless 227
low density lipoprotein receptor 294
low density lipoprotein receptor-related protein (LRP) 84, 294, 297, 298
lysosomes 136

M

Madin-Darby canine kidney (MDCK) cells 138
Menkes disease 79
metal-ion binding 77
MG132 121
micelles 171, 172, 175
micellization 171, 175
microtubule associated protein (MAP) 17
microtubule associated protein 1 A (MAP1A) 23
microglia 7, 314
microtubule 20
missense mutations 57
missorting 139
mixed brain cultures 236
molecular genetics 47
monensin 120
MTT assay 252, 253, 265
muscarinic receptor 281

N

N2a cells 156

NAC (see non-amyloid component)

necrosis 249
neurites 314
 dystrophic neurites 314, 319
 outgrowth 295
neurodegeneration 142
neurofibrillary tangles (NFT) 9, 17, 163, 294, 319
 argyrophilic NFTs 27, 28
neurofilament 314
neurons 120, 141, 316
neuropil threads 28
neuroprotection 86, 87
neurotoxicity 87
NF κ B 247, 260–271
non-amyloid component (NAC) 3
non-neuronal cells 120
NOTCH 56, 199, 222, 226, 227, 229, 260
Northern blot 209
nucleation 165, 187
nucleation rate 170, 173, 175

O

olfactory nucleus 311
overexpression 196, 200
oxidation 267, 268
oxidative stress 87

P

p3 96, 97, 99, 100, 102, 239
pathogenesis 318
PEST motif 278, 282
PHF1 316
phorbol ester 98, 263
phosphatases 34
phosphoepitopes 316
phosphorylation 25, 277, 281
plasma 85, 155, 156
pleiotropic effects 311
polarized protein transport 133
polymer size 175
polymerization 165
polymerization kinetics 165

positional cloning 48, 54
 prenucleation 172, 187
 presenilin 61, 103, 114, 193–201, 247, 253
 conditional targeting 244
 double knock out (PS1 and PS 2) 242
 DPS (Drosophila PS) 220, 221, 225
 expression 222
 function 230
 knock out 227, 236
 mutation 225
 overexpression 231
 presenilin 1(PS1) 49, 54, 114, 154, 155, 156, 193–201, 207, 219–222, 227, 235, 253, 255, 256
 presenilin 2 (PS2) 49, 59, 114, 154–156, 193, 207, 219–222, 227, 235, 253, 255–257, 263, 265, 266, 271
 presenilin knock out 200
 phenotype 237
 proteolytic processing/endoproteolysis 195–199, 279, 281
 topology 194, 208
 Xenopus PS (X-ps-a) 220, 221, 225
 Xenopus PS (X-ps-b) 220, 221, 225
 presymptomatic carriers 155
 programmed cell death 249
 proline-directed protein kinase (PDPK) 18
 proline-rich region 22
 promotor 310
 protease nexins 83
 protein kinases 277
 protein kinase A (PKA) 18, 277, 280
 protein kinase C (PKC) 18, 98, 277, 280
 protein phosphatases 277
 proteasome 122
 proteoglycans 82
 proteolytic fragments 210
 protofibrils 181, 183, 184, 187
 protofibril formation 182, 185, 186
 pulse-chase 103

Q

Quasielastic light scattering spectroscopy (QLS) 167, 177, 178

R

rab5 144, 145, 147
 racemization 25
 radial organization 237
 radical based APP fragmentation 80
 radiosequencing 102
 reactive free radicals 156
 reactive microglia 156
 reactive oxygen species 80
 RT-PCR 210

S

sandwich ELISA 154, 241
 Scavenger receptor A 297
 secretase 230
 α -secretase 97, 113, 134, 241
 β -secretase 99, 113, 121
 γ -secretase 99, 113, 121, 201, 242, 243
 seeds 159, 172
 sel 12 55–59, 194, 208, 220–230
 egg-laying 222, 223, 228, 230, 231
 expression 222
 function 226, 230
 mutation 225, 228
 promotor 231
 Semliki Forest virus 117, 128, 138, 239
 signaling pathways 280
 site-2 protease (S2P) 243, 244
 size exclusion chromatography (SEC) 177, 178, 181–184, 186, 187
 somite 227
 somitogenesis 200
 spe 4 55, 208, 220–222, 226, 230
 expression 222
 fibrous body membranous organelle (FBO) 226
 function 226, 230
 mutation 225
 sperm development 226
 spinocerebellar ataxia typ 3 123, 124, 129
 sporadic AD 61, 65, 67, 155–159, 235–236, 244
 SREBP cleavage activating protein (SCAP) 242
 sterol regulatory element binding protein (SREBP) 242

subcellular trafficking 139, 140
 subiculum 311
 susceptibility locus 52
 synapses 319
 synaptic plasticity 75, 77
 synaptophysin 314

T

tau 11, 12, 14, 17, 316, 318–323
 terminal UTP-nick end labeling (TUNEL) 252, 253, 255, 256
 thalamus 311, 312
 Thioflavin S 311, 312
 TNF α 257, 258, 260–266
 toxic effects 156, 157
 transectosis 143, 147
 transfected cells 97, 98, 100, 102, 105, 154, 156, 159
 transgenic mice 101, 102, 105, 109, 156, 196, 309–311, 313, 314, 316–329
 behavioral changes 319
 transglutaminase 30, 37
 transport 226, 229, 230, 231
 transthyretin 10, 14
 trisomy 21 154

U

ubiquitin 25, 26, 27, 35, 38, 40, 41, 122
 ubiquitination 25, 26, 27, 35, 41

V

vascularization 237
 video-microscopic analysis 22
 Volga-German kindred 207

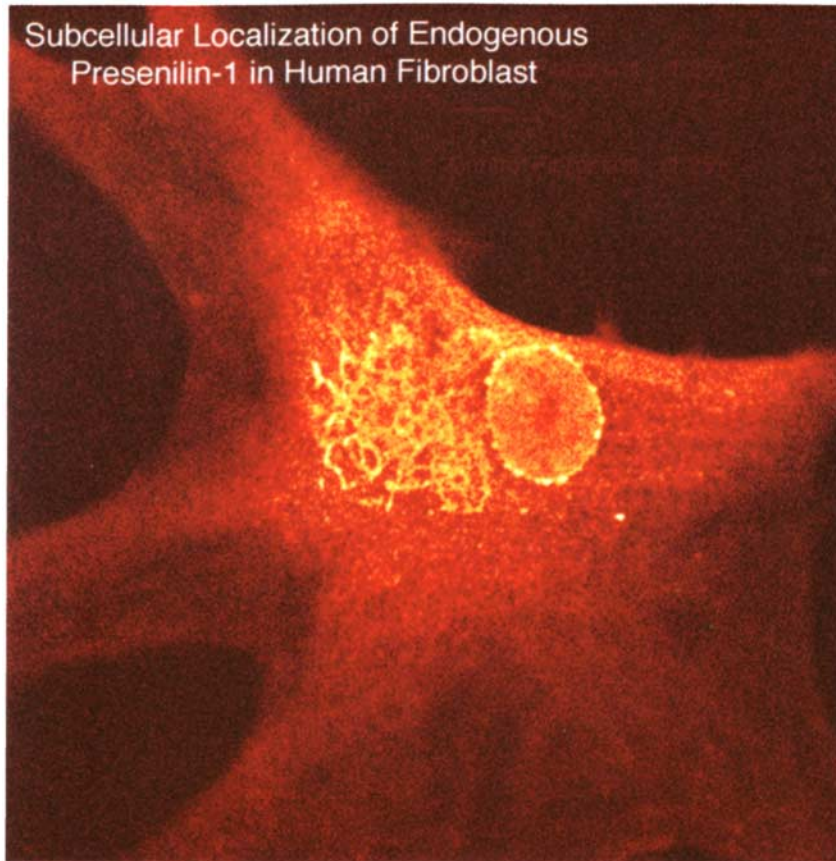
W

white matter 4, 8, 12
 Wilson disease 79, 89
 wingless 229, 230, 232

Z

Zinc 77
 zinc(II) binding site 82, 88

Subcellular Localization of Endogenous
Presenilin-1 in Human Fibroblast



COLOUR PLATE I. See P.E.Fraser and P.H.ST George-Hysop, [Figure 1](#), page 56.

Hu PS1	1	MTPELPALSY FQNAQMSQDN HL.....SNT VRQNDNRRER QEHNDRRSLG	45	
Xl PSa	1		MND.....TSERRSNE	11
Hu PS2	1	ML TFMASDSEKE VCDERTSLMS ABS.PTPRSC QEGR.....	35	
Xl PSb	1	MIKLSDSEDE ECNERTSLIT SES.PPLPSY QDGV.....	33	
Dm PS	1	MAAVNLQAS CSSGLASEDD ANVGSQIGAA ERLERPPRRQ QORNNYGSSN	49	
Ce sel-12	1		MPSTRQ QEGGG.....	12
Ce spe-4				

Hu PS1	46	HPEPLSNGRP QGNSRQ.VVE QDEBE.....	DEEL	73
Xl PSa	12	NSESQSNQQT QSSSQ.VLE QDEBE.....	DEEL	39
Hu PS2	36	QGPED GENTAQRNS. QENBEDGEED PDRYVCSGVF GRPFGLEEL	79	
Xl PSb	34	QAASEGLETS YHRERQPDST QNNEOVPNGR TSGADAYNSE TTVENEEEL	82	
Dm PS	50	QDQPD AILAVPNVVM REPCCSRPSR LTGGGGGSGG PPTNEMEBEQ	95	
Ce sel-12	13	ADAETHTVY GTNLITNRNS QEDENV.....	VEEA	41
Ce spe-4	1		MD TLR...ISS EL....VRSS	15

		79V 81L	60S	115C.H	120D.K		
Hu PS1	74	TLKYGAKHVI	MLFVFTLCM	VVVVATIKSV	SFYTRDGG.Q	LI.TPFT.DT	122
Xl PS1	40	TLKYGAKHVI	MLFVFTLCM	VVVVATIKSV	SFYTRDGG.Q	LIYTPFTEDT	88
Hu PS2	80	TLKYGAKHVI	MLFVFTLCM	IVVVATIKSV	RFYTEKNG.Q	LIYTPFTEDT	128
Xl PS2	83	TLKYGAKHVI	MLFVFTLCM	VVVVATIKSV	SFYTRDGG.Q	LIYTPFTEDT	131
Dm PS	96	GLKYGASHVI	HLFVFTLCM	LVVVATIKSV	SFYNSTEV.Y	LIYTPFTEDT	144
Ce sel-12	42	GLKYGASHVI	HLFVFTLCM	ALVVFTIKSV	TFYSQNNGRH	LIYTPFTEDT	91
Ce spe-4	16	QRWTLFSVI	ANM.SLTLSI	WICVYNMEVN	SELSKT...Y	FLD.PSFEQ	60

		141I	135D	139I.V.T	143F.T	146I.L.V	163R.Y	
Hu PS1	123	ETVGQRPLNS	ILNATIMISV	IVVTILLVV	LYKYRCYKVI	KAWLIHSSLL		172
Xl PS1	88	ESVGQRPLNS	ILNATIMISV	IVVTILLVV	LYKYRCYKVI	HGWLHSSLL		138
Hu PS2	129	PSVGQRPLNS	VNLNIMISV	IVVTILLVV	LYKYRCYKVI	HGWLHSSLL		178
Xl PS2	132	TSVGQRPLNS	VNLNIMISV	IVVTILLVV	LYKYRCYKVI	HGWLHSSLL		181
Dm PS	145	PEPSVKFWSA	LANSILIMSV	VVMTILLIV	LYKYRCYKVI	HGWLHSSLL		194
Ce sel-12	91	DSIVEKGLNS	LGNATIMISV	VVMTILLIV	FYKYRCYKVI	HGWLHSSLL		141
Ce spe-4	61	T..GNLLLDG	FINGVGTILV	LGCVSFIMLA	FVLEDFRRIV	KAWLTSSLL		108

		209V	213T				
Hu PS1	173	LLF.....	..FESFYILG	EVKRTYNVAV	DYITVALLIV	NFGVVMIS	213
Xl PS1	139	LLF.....	..FESFYILG	EVKRTYNVAV	DYITVALLIV	NFGVVMIS	179
Hu PS2	179	LLF.....	..LFTYIYLG	EVKRTYNVAV	DYITVALLIV	NFGVVMIS	219
Xl PS2	182	LLF.....	..MFTYIYLS	EVKRTYNVAV	DYITVALLIV	NFGVVMIS	222
Dm PS	195	LLF.....	..IFTYIYLE	ELLRAYNIFM	DYITVALLIV	NFGVVMIS	235
Ce sel-12	142	LLF.....	..LFTYIYVQ	EVKRTYNVAV	SALLVLFGLG	NFGVVMIS	182
Ce spe-4	109	ILFGVSAQTL	HDMESQVFDQ	DDNNOYMTI	VLIIVPTVVY	GFG..GIYAF	156

		231T.V	233T	235P	246E	250S	225Stop	260V	263R
Hu PS1	214	HWKGPLRLQQ	AYLIMISALM	ALVFIKYLPE	WT.WLILAVH	SVYDLV.VLC			263
Xl PS1	180	HWKGPLRLQQ	AYLIMISALM	ALVFIKYLPE	WT.WLILAVH	SVYDLVAVLS			229
Hu PS2	220	HWKGPLRLQQ	AYLIMISALM	ALVFIKYLPE	WSAWVILGAE	SVYDLVAVLC			269
Xl PS2	223	HWKGPLRLQQ	AYLIMISALM	ALVFIKYLPE	WSAWVILGAE	SVYDLVAVLC			272
Dm PS	236	HWKGPLRLQQ	AYLIMISALM	ALVFIKYLPE	WTAWVILGAE	SVYDLVAVLS			285
Ce sel-12	183	HWKGPLRLQQ	AYLIMISALM	ALVFIKYLPE	WTAWVILGAE	SVYDLVAVLS			232
Ce spe-4	157	FSNSSLILHQ	IFVVTNCSLI	SVFYLRVFS	RTAWVILGAE	LFWDLPAVLA			206

267S
264L 269GH 278T280A.G 286V 289S 285V
Δexon10 (hg347)

Hu PS1 264 KG L MLVE TAQRNNTTF PALIYSSMV ...MNV... NMAAGGFEA 305
X1 PS1 230 PKGPLRLMLVE TAQRERNNTTF PALIYSSSMI ...MNV... NMAAGDGGL 271
Hu PS2 270 PKGPLRLMLVE TAQRERNNTTF PALIYSSSMV ...MNV... CMAKLDESS 311
X1 PS2 273 PKGPLRLMLVE TAQRERNNTTF PALIYSSSMV ...MNV... CMAASATA. 313
Dm PS 286 PKGPLRLMLVE TAQRERNNTTF PALIYSSIVV YALVNTVTQP QQAQASSSP 330
Ce sel-12 233 PKGPLRLMLVE TAQRERNNTTF PALIYSSGVI YPYVNTVAVE NITDDEBS... 285
Ce spe-4 207 PKGPLKKVQE KASDYSKCVL N LMFSSANE KRLTAGSNQE ETNEGEESTI 255

318G (hg347)

Hu PS1 306 QRRVSSNSKY NA ...STERESQ DTVAENDDGG FSEEFQARD 345
X1 PS1 272 KQASAKTK.Y NIT... ..APTAAHPS DSAASDNDGG FDTTWEDHRN 311
Hu PS2 312 ...QGAL... ..LPLY... ..D.PEMEDS. YDSFGE... 333
X1 PS2 314 ...DG... ..RM N... ..QVQHI D...RNTPE.GA...NSTVE... 337
Dm PS 336 SSSNSTTTTR ATNSLASPE AAAASGORTG NSHPQRQND DGSVLA... 381
Ce sel-12 281 TSSDSNTSTA FPGEASCSE TPKRPVKKRI PQKVQIESNT TASTTQN... 327
Ce spe-4 256 RRTVKQTIEY YTKREAQDDE FYQKIRQRR AINPDSVPE HSPLVEAAEPA305

Hu PS1 346 SHLGPH.RST PESRAAVQEL SSSILAGDPP ... 374
X1 PS1 312 AQIGPI.NST PESRVAVQAL PMSPPSPDP ... 340
Hu PS2 334 ...PSY PEVFEPLPTG YPGEELE... 355
X1 PS2 338 ...DA. AETRIQTQ... ..SNLSS...DPP D... 356
Dm PS 382 ...TEGMPLVTFFK... ..SNLRGNAE AGFTQEWAN LSERVARROI 420
Ce sel-12 328 ...SGVRV...RE LAAERPTVQD ANFHRHEE... 353
Ce spe-4 306 SPIELKEKNS TEELSDDSD TSTSSGGSN LSSSDSTTV STDISTAE 355

384A 392V

Hu PS1 375 ... EERGVKLGL DFIFYSV VG 394
X1 PS1 341 ... EERGVKLGLG DFIFYSVLVG 360
Hu PS2 356 ... EERGVKLGLG DFIFYSVLVG 375
X1 PS2 357 ... EERGVKLGLG DFIFYSVLVG 376
Dm PS 421 EVQSTQSGNA QRSNEYRTVT APQNHPPQG EERGVLKGLG DFIFYSVLVG 370
Ce sel-12 354 ... EERGVKLGLG DFIFYSVLVG 473
Ce spe-4 357 CDQEKWDDLIV SNLSLNNDRK PATAADALND GE...LRLGFG DSVFYSDIG 403

381Stop 410Y 426P 436S

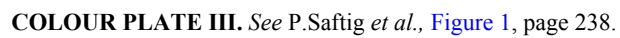
TM7

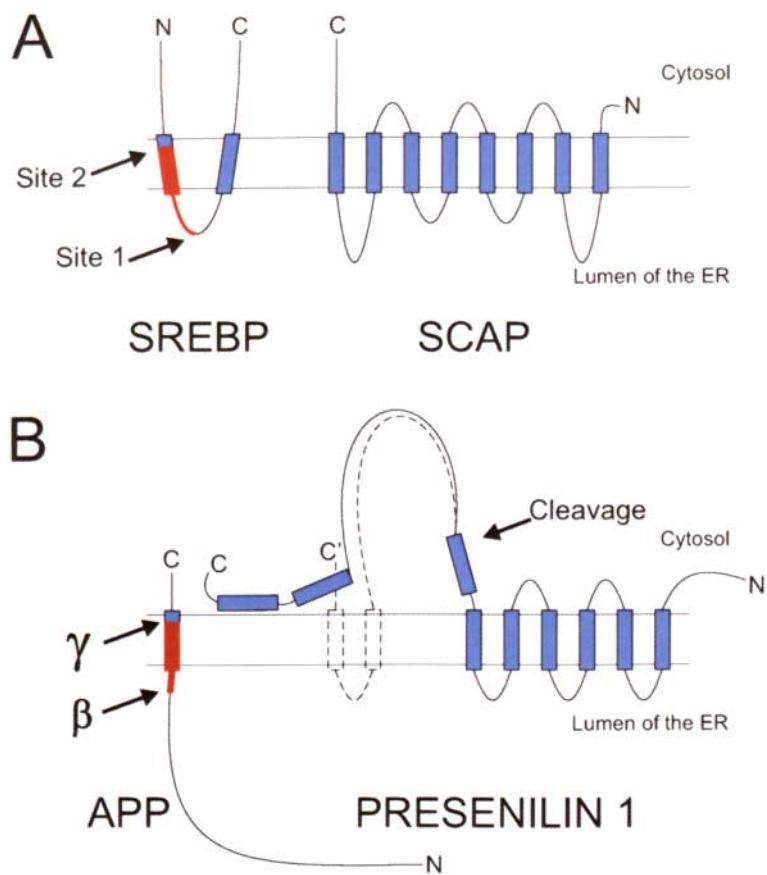
Hu PS1 395 KASATASGDW NTTIA FVAI LIGLCLTLL L LKKKALPA L LISITFGLV 444
X1 PS1 361 KASATASGDW NTTIACFVAI LIGLCLTLL L LKFKKALPA LPISITFGLV 410
Hu PS2 376 KASATASGDW NTTIACFVAI LIGLCLTLL L LKFKKALPA LPISITFGLI 425
X1 PS2 377 KASATASGDW NTTIACFVAI LIGLCLTLL L LKFKKALPA LPISITFGLI 426
Dm PS 471 KASSY...GDW NTTIACFVAI LIGLCLTLL L LKFKKALPA LPISITFGLI 518
Ce sel-12 374 KASSYF...DNTTIIACFVAI LIGLCLTLL L LKFKKALPA LQFFSPSPF 421
Ce spe-4 404 GAA...GCCP FAV...SAALG LIGLCLTLL L LKFKKALPA LPLFVICITF 451

TM8 TM9

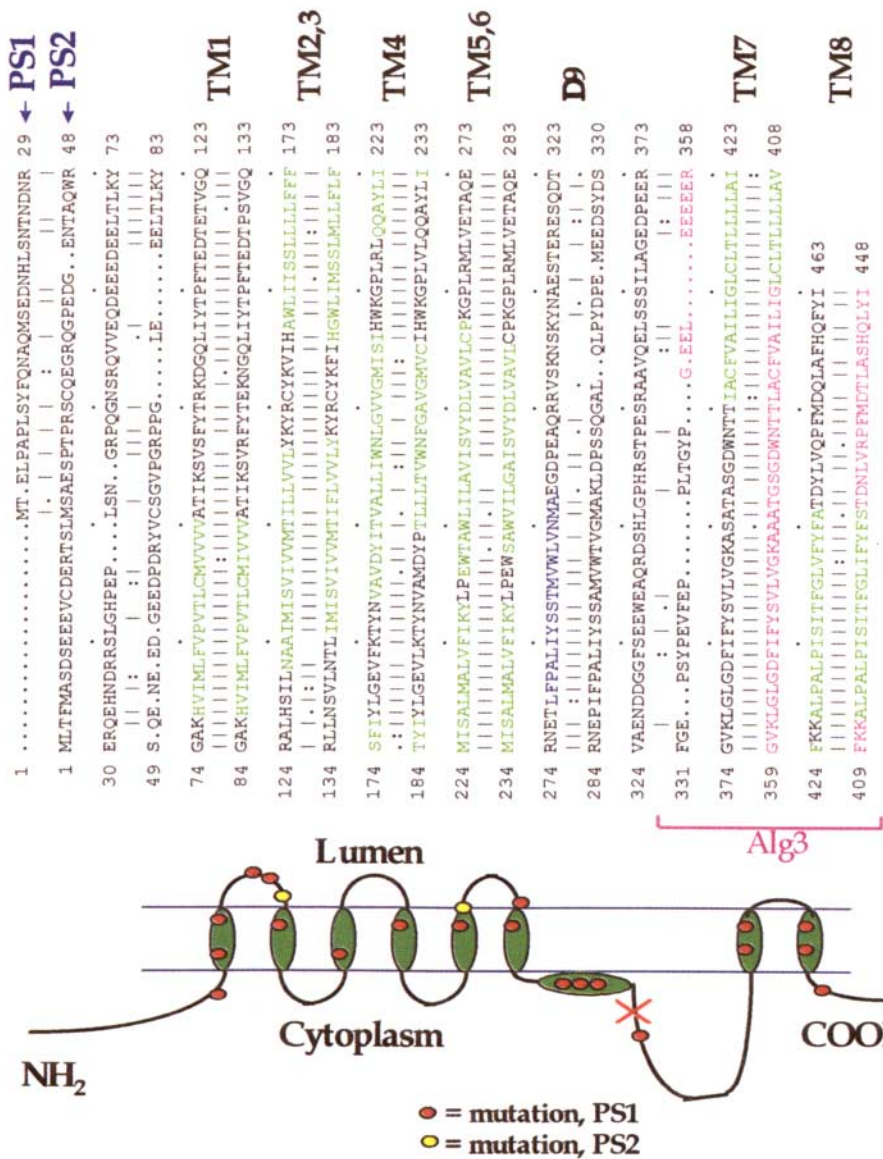
TM9

Hu PS1 445 FYFATDYLVO PFMDQLAFH...PFYI 467
X1 PS1 411 FYFATDYLVO PFMDQLAFH...PFYI 433
Hu PS2 426 FYFSTDNLVR PFMDQLASH...QLYI 448
X1 PS2 427 FYFSTDNLVR PFMDQLASH...QVYI 449
Dm PS 519 FYPASAVVX PFMDLSAK...QVYI 541
Ce sel-12 422 FYFGRWIIT PFVTVSQKC LLV 444
Ce spe-4 452 CFFSSMFME ... 465

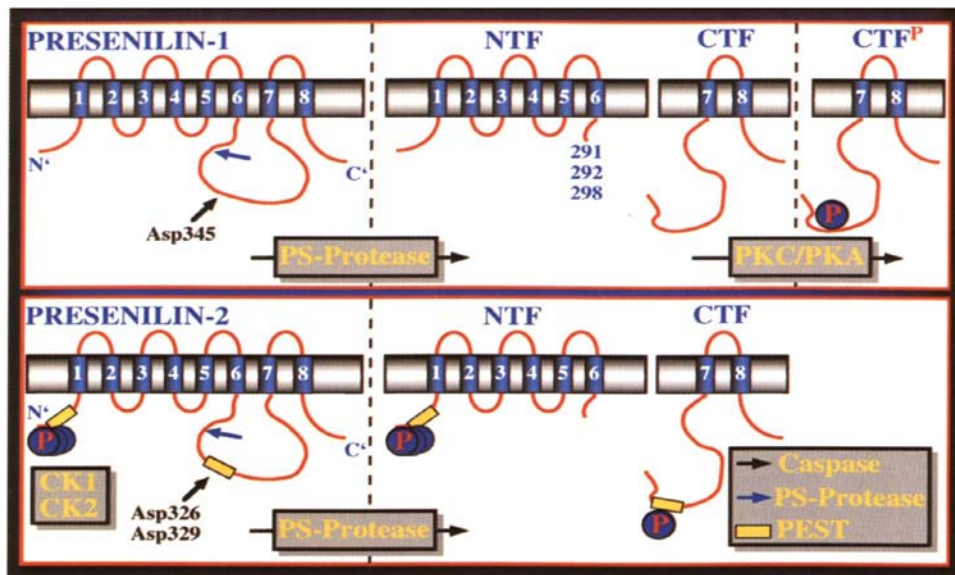




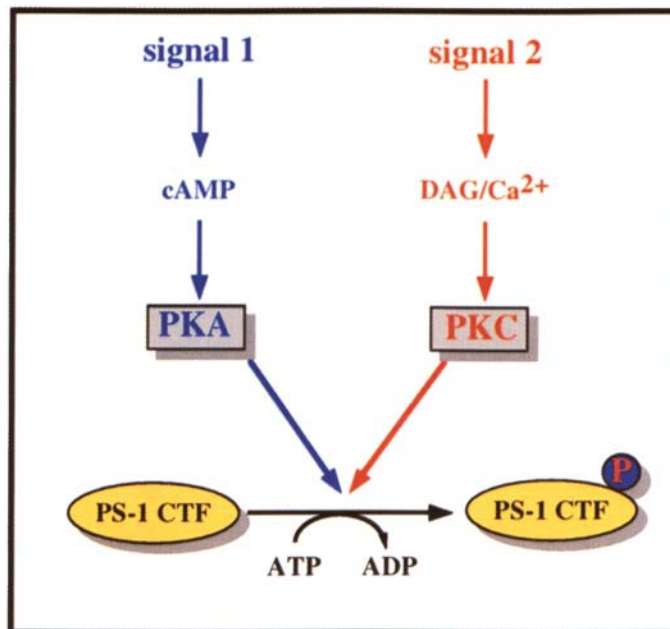
COLOUR PLATE IV. See P.Saftig *et al.*, [Figure 4](#), page 243.



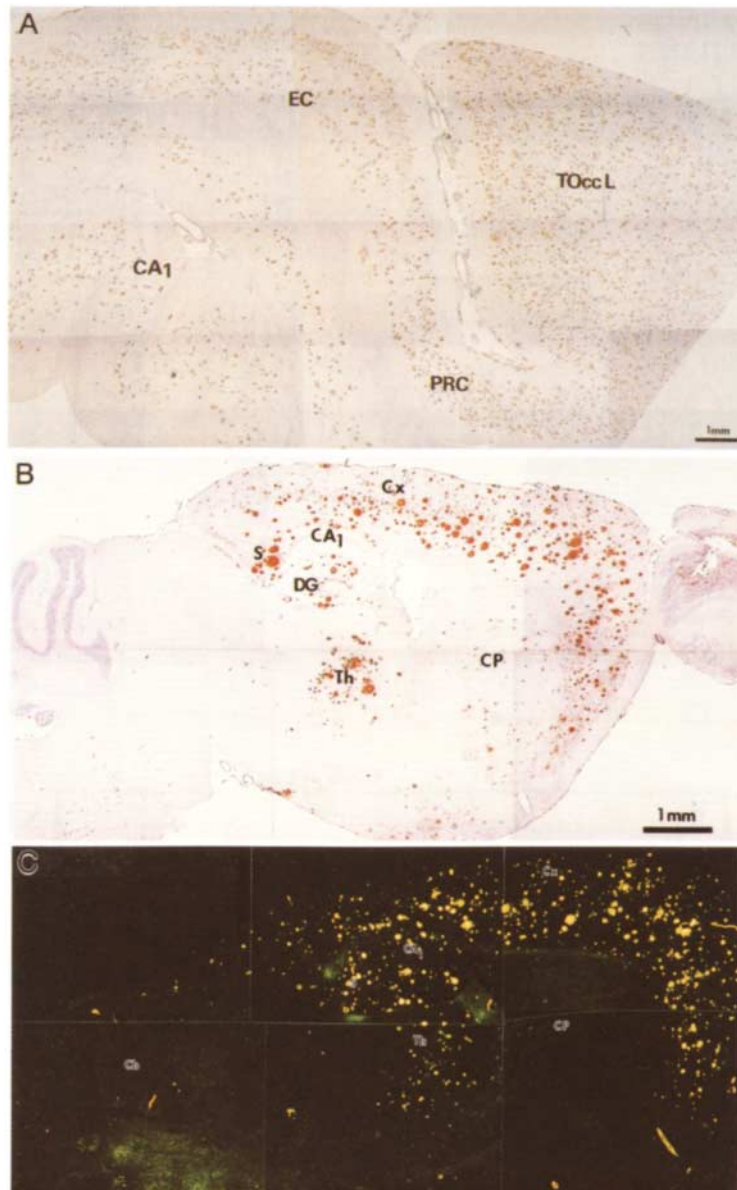
COLOUR PLATE V. See B.Wolozin and J.Palacino, [Figure 2](#), page 254.



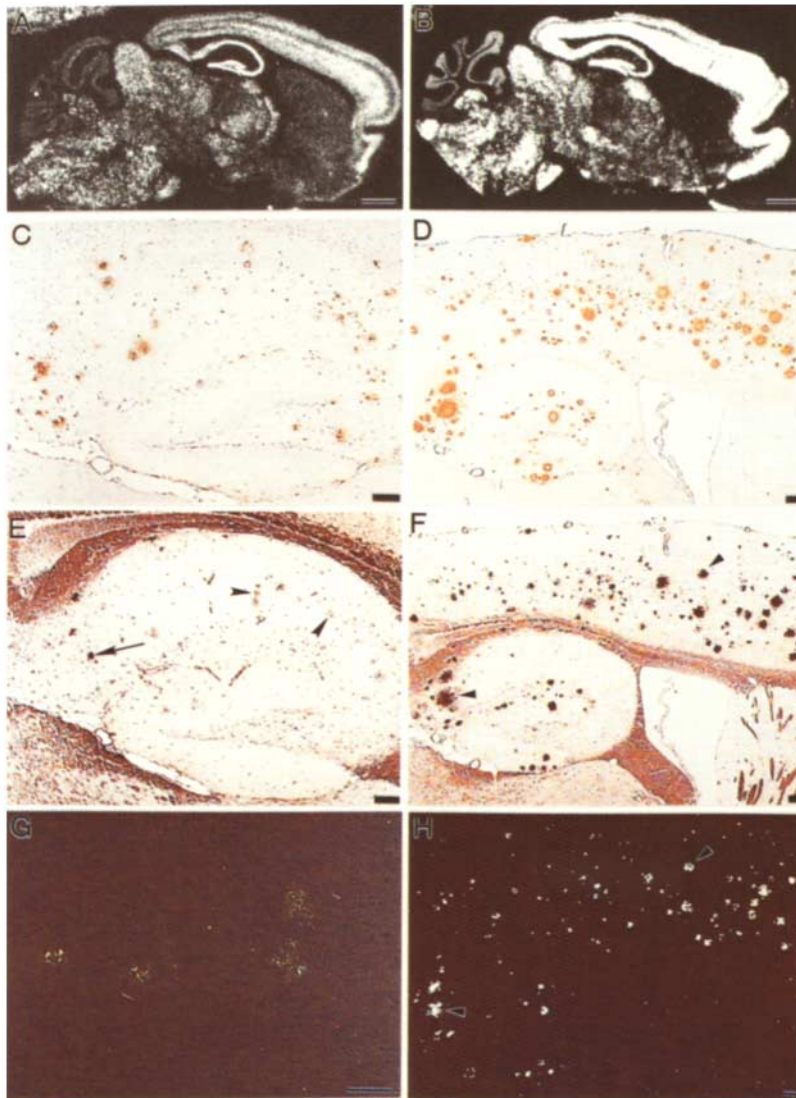
COLOUR PLATE VI. See J.Walter and C.Haass., [Figure 2](#), page 279.



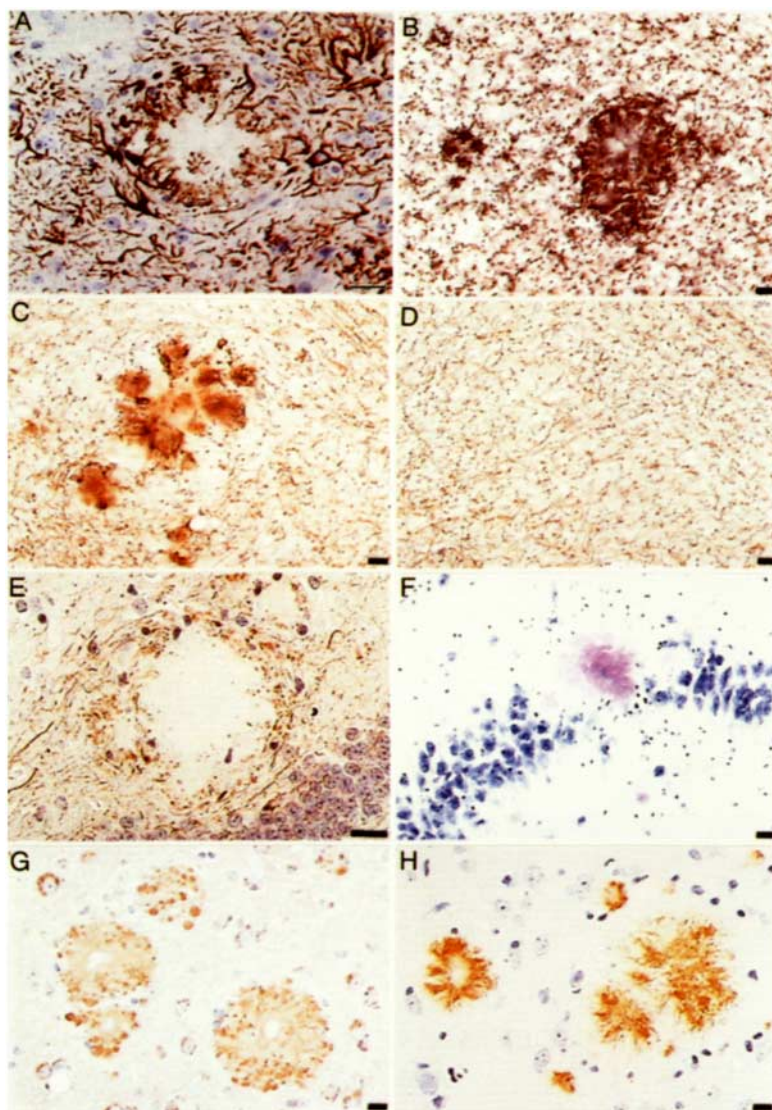
COLOUR PLATE VII. See J.Walter and C.Haass., [Figure 3](#), page 280.



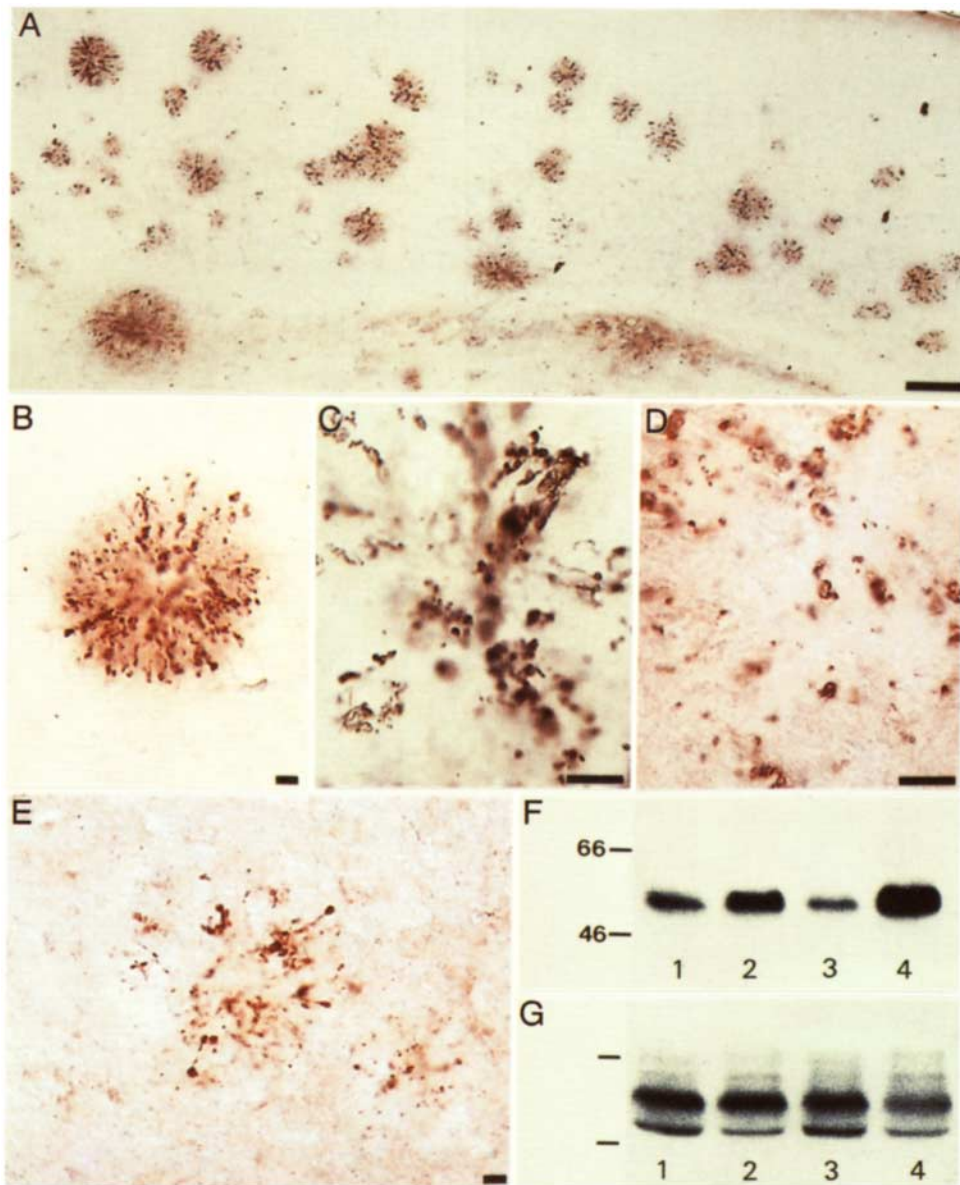
COLOUR PLATE VIII. See M. Staufenbiel and B. Sommer, [Figure 1](#), page 312.



COLOUR PLATE IX. See M.Staufenbiel and B.Sommer, [Figure 2](#), page 313.



COLOUR PLATE X. See M.Staufenbiel and B.Sommer, [Figure 3](#), page 315.



COLOUR PLATE XI. See M.Staufenbiel and B.Sommer, [Figure 4](#), page 317.