AUTISM: NEUROPATHOLOGY, ALTERATIONS OF THE GABAERGIC SYSTEM, AND ANIMAL MODELS

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Autism is a neurodevelopmental disorder with a strong genetic component and several known environmental risk factors. Neuropathological studies have shown consistent abnormalities in the limbic system, cerebellum, and cerebral cortex. Several findings suggest a role for the GABAergic system in autism

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neuropathology. There are reports of elevated plasma GABA levels, reduction of the GABAergic system enzymes, and decreased availability of GABA in autistic patients. Autism has a reported heritability of 60–90%. Abnormalities in the 15q11–13 region have been found in autistic people, and the $GABA_A$ receptor genes are located in this region. In addition, GABA dysfunction may occur in conjunction with Reelin. Abnormalities in the gene encoding for Reelin have been implicated in autism, and Reelin and GABA play an important role in the development of minicolumns. Compared to controls, minicolumns are more numerous, smaller, and less compact in autistic patients. Several studies provided evidence for the role of GABA receptors in tangential migration of neurons. Furthermore, GABA regulates cell proliferation in some brain regions. Because the underlying causes of the reduced GABA system function in autism are not well understood, it is important to develop animal models of autism, which can give more insights into the neuropathology and behavioral aspects of the disease. Animal models of autism include misregulation of genes implicated in the disorder, as well as the use of known environmental risk factors. In the future, investigation of human autism tissues and animal models, in combination with implementation of new techniques such as design-based stereology and gene expression, may result in the elucidation of the etiology of autism.

I. Introduction

Autism is currently viewed as a genetically determined neurodevelopmental disorder (Bailey *et al.*, 1996), defined by the presence of marked social deficits, specific language abnormalities, and stereotyped, repetitive behaviors (American Psychiatric Association, 1994). Approximately 20% of the autistic subjects show macroencephaly, defined as head circumference above the 97th percentile (Aylward *et al.*, 2002; Bailey *et al.*, 1993; Courchesne *et al.*, 2003; Davidovitch *et al.*, 1996; van Karnebeek *et al.*, 2002). However, this macroencephaly is not present until after the first year of life (Courchesne *et al.*, 2003). Although evidence of increased head circumference (Aylward *et al.*, 2002; Bailey *et al.*, 1993; Courchesne *et al.*, 2003; Davidovitch *et al.*, 1996; Fombonne, 2000; van Karnebeek *et al.*, 2002), brain weight (Bailey *et al.*, 1998; Casanova *et al.*, 2002b; Courchesne *et al.*, 1999; Kemper and Bauman, 1998), and brain volume (Aylward *et al.*, 2002; Courchesne *et al.*, 2001; Sparks *et al.*, 2002) has been described in autism, the underlying biological mechanisms remain to be determined. These observations could be due to increased neurogenesis, gliogenesis, or synaptogenesis, disturbed neuroblast migration, decreased apoptosis or synaptic pruning, or combinations of these effects (Palmen *et al.*, 2004). In addition, the detection of abnormalities in neurotransmitter systems, such as γ-aminobutyric acid (GABA) in autistic

patients, suggests that it could be worthwhile to concentrate research on these systems. A good approach is the use of animal models that mimic features of autism.

In this chapter we examine the literature on autism neuropathology, the role of the GABAergic system in this disorder, and the relevance of rodent models with autistic features.

II. Neuropathologic Alterations in Specific Brain Regions in Autism

A. Limbic System

Bauman and Kemper were the first to report neuropathological findings in autism. They demonstrated increased cell packing density and smaller neurons in several regions of the limbic system, including the hippocampus, subiculum, amygdala, entorhinal cortex, mammillary bodies, and septal nuclei (Bauman, 1991; Bauman and Kemper, 1985, 1987, 1990; Bailey *et al.*, 1998; Kemper and Bauman, 1993; Raymond *et al.*, 1996). This pattern of small, closely packed neurons with reduced dendritic arbors could reflect features of an immature brain (Jacobsen, 1991). Bailey *et al.* (1998) demonstrated increased cell packing density in all CA regions of the hippocampus in one out of five autistic cases. Raymond *et al.* (1996) investigated the dendritic morphology of hippocampal neurons in two well-documented autistic patients and one age-matched control. Using the Golgi stain, this group found smaller neurons and less dendritic branching of both CA1 and CA4 hippocampal neurons in autistic subjects compared to controls. These findings further suggest a curtailment of maturation, a feature previously highlighted by Kemper and Bauman (1993).

Neuropathologic findings are rather consistent with respect to the limbic system, whereas MRI studies are mostly conflicting. For example, compared to controls, volumes of the limbic system of autistic subjects are reported to be increased (Howard *et al.*, 2000; Sparks *et al.*, 2002), decreased (Aylward *et al.*, 1999), or unchanged (Howard *et al.*, 2000; Piven *et al.*, 1998).

B. Cerebellum and Brainstem

Williams *et al.* (1980) were the first to examine neuropathological alterations in the cerebellum in autism. In one out of four brains from autistic patients they found reduced Purkinje cell density. In a study by Ritvo *et al.* (1986), all autistic cases showed a decreased number of Purkinje cells in the cerebellar vermis and hemisphere. Kemper and Bauman (1993) replicated this finding. In another

study five adult autistic cases had a low number of cerebellar Purkinje cells, although this feature was not found in a 4-year-old autistic male (Bailey *et al.*, 1998). In contrast, no abnormalities were found in the cerebellum of a 16-year-old female with autism and severe mental retardation (Guerin *et al.*, 1996). Because of the consistent findings of decreased numbers of Purkinje cells in the cerebellum, Fatemi *et al.* (2002b) examined the size of these cells. They showed a 24% decrease in mean Purkinje cell size in autistic brains. It should be mentioned that cerebellar Purkinje cells are the final targets of projections from the inferior olivary nucleus. A decrease in Purkinje cells may result in an abnormal development of the olivary projections to the cerebellar nuclei (Palmen *et al.*, 2004). Many research groups have found a decrease in the number of cerebellar Purkinje cells without significant gliosis in the cerebellum (Bailey *et al.*, 1998; Fatemi *et al.*, 2002b; Kemper and Bauman, 1993). Most MRI studies have shown smaller cerebellar hemispheres (Courchesne, 2004; Murakami *et al.*, 1989) or vermis (Courchesne, 2004; Hashimoto *et al.*, 1995; but see Nowell *et al.*, 1990; Piven *et al.*, 1997).

Furthermore, two out of six brains from autistic patients in the study of Kemper and Bauman (1993) demonstrated enlarged neurons in deep cerebellar nuclei and inferior olivary nucleus, whereas in the older subjects (>22 years) these neurons were small and pale, with normal numbers. In all brains, the inferior olivary nucleus did not show retrograde loss of neurons. Moreover, age-related abnormalities in the cerebellar nuclei and the inferior olive have been reported (Bauman, 1991). Apart from reports on limbic alterations, Bailey *et al.* (1998) have demonstrated olivary dysplasia in three of five autistic cases. In another two autistic subjects they found ectopic neurons related to the olivary complex (Bailey *et al.*, 1998). Finally, Rodier *et al.* (1996) reported that the brain of a 21-year-old autistic woman with mental retardation and comorbid epilepsy, exhibited near-complete absence of the facial nucleus and superior olive along with shortening of the brainstem between the trapezoid body and the inferior olive.

C. Migration Abnormalities and Cortical Dysgenesis

Reelin is a signaling molecule that plays a role in migration and lamination of neurons during embryogenesis. In adult life Reelin is involved in synaptic plasticity (Fatemi, 2000b, 2002, 2004). In the cerebellar cortex, Fatemi *et al.* (2001a,b) found a >40% reduction in Reelin and a 34–51% reduction in Bcl-2 levels in autistic subjects. Very recently, Fatemi *et al.* (2005) observed reduced Reelin signaling in the frontal cortex in autism. There is an association of decreased Reelin levels with disturbed neuronal migration and lamination of the cerebral and cerebellar cortex in mice (Gonzalez *et al.*, 1997) and humans

(Persico *et al.*, 2001; Piven *et al.*, 1990). Furthermore, decreased Reelin levels in the blood have been related to severe mental retardation and hypoplastic cerebellum, both of which have been reported in autism. The decreased Bcl-2 levels might inhibit apoptosis. Both decreased Bcl-2 and increased P53 levels were correlated to mental retardation and have been suggested to result in a greater propensity for cell death (Fatemi and Halt, 2001a). Cortical dysgenesis was identified in four of six (all mentally handicapped) autistic cases (Bailey *et al.*, 1998). These brains showed thickened cortices, high neuronal density, presence of neurons in the molecular layer, irregular laminar patterns, and poor gray-white matter boundaries.

Given the importance of cortical cellular organization during development, Casanova *et al.* (2002a) investigated the morphology of cell minicolumns. Compared to controls, there were more numerous, smaller, and less compact minicolumns in autistic subjects (Casanova *et al.*, 2002a,b). However, the functional significance of these changes in minicolumns is still unclear (Hutsler *et al.*, 2003).

D. Cholinergic System

Hohmann *et al.* (1998) emphasized the importance of the cholinergic system during brain development. They reported a delay in cortical development, permanent changes in cortical architecture, and cognitive function when cholinergic innervation is disrupted during early postnatal development. Furthermore, Bauman and Kemper (1994) demonstrated larger cholinergic neurons in young autistic cases compared to smaller cholinergic neurons at an older age. Perry *et al.* (2001) investigated levels of cholinergic enzyme and receptor activities in the frontal and parietal cerebral cortex. Compared to non-autistic mentally retarded cases, the autistic cases display 30% lower muscarinic M1 receptor binding in the parietal cortex. In addition, a reduction in $\alpha 4$ nicotinic receptor binding was found in all groups as compared to controls. In the cerebellum, the nicotinic receptor ($\alpha 3$ and $\alpha 4$ subunit) was reduced by 40–50%, whereas the $\alpha 7$ subunit was increased in autism (Lee *et al.*, 2002).

III. GABAergic Abnormalities in Autism

The GABAergic system has also been suggested to be involved in autism (Blatt *et al.*, 2001; Cook *et al.*, 1998; Schroer *et al.*, 1998). GABA is implicated in various psychiatric disorders, including schizophrenia (Caruncho *et al.*, 2004; Guidotti *et al.*, 2000), mood disorders (Sanacora *et al.*, 1999), anxiety disorders

(Goddard *et al.*, 2001), and autism (Cook *et al.*, 1998; Schroer *et al.*, 1998). Changes in GABA levels have been found in platelets of 18 of 18 autistic children (Rolf *et al.*, 1993) and in the plasma and urine of one male (case study) autistic child (Cohen, 1999, 2000). Dhossche *et al.* (2002) hypothesized that the elevated plasma levels of GABA could reflect a compensatory increase in presynaptic GABA release in response to hyposensitivity of a subset of GABA receptors. In turn, this could produce increased postsynaptic activation of other, normal GABA receptors subtypes, resulting in complex alterations of GABAergic function throughout the brain in autistic people. By introducing a GABA-transaminase agonist, the GABA plasma levels can be lowered (Cohen, 2002). During normal GABA catabolism, GABA-transaminase is responsible for the conversion of GABA into succinic semialdehyde (SSA) (Tillakaratne *et al.*, 1995). Such a GABA-transaminase agonist can activate GABA-transaminase enzyme activity by causing a reduction of plasma GABA levels in the brain. They proposed that this could result in an elevated signaling between axons and oligodendrocytes in the corpus callosum, which might result in a decrease of autistic features due to abnormal development of axons in the corpus callosum (Cohen, 2002). Cohen (2000) also reported increased levels of ammonia in the liver of the same autistic child, illustrating a possible link between the liver and infantile autism.

Blatt *et al.* (2001) investigated the hippocampal density and distribution of neurotransmitter receptors from the GABAergic, serotonergic (5-HT), cholinergic, and glutamatergic systems in autistic patients and controls (age range 16–24 years). They reported that only the GABAergic receptor system was significantly reduced in autism. It has been suggested that some autistic patients respond abnormally to benzodiazepines, possibly due to pre-existing GABAergic dysfunction or abnormalities in the GABA benzodiazepine receptor complex (Garreau *et al.*, 1993). Furthermore, ^{3}[H]-flunitrazepam-labeled benzodiazepine binding sites and ^{3}[H]-muscimol labeled $GABA_A$ receptors were reduced in the hippocampus of autistic people (Blatt *et al.*, 2001), which provides evidence for abnormal benzodiazepine receptor complexes in the hippocampus of these people. Fatemi *et al.* (2002c) found reduced levels of both glutamic acid decarboxylases (GAD)65 and GAD67 in autistic parietal and cerebellar cortex, implying a deficit in GABA. Lower GABA levels could reduce the threshold for developing seizures, which are often associated with autism (Bailey *et al.*, 1998; Blatt *et al.*, 2001).

The heritability of autism is 60–90% (Andres, 1998; Bespalova and Buxbaum, 2003). Abnormalities on the long arm (15q11–13) of the maternally derived chromosome 15 have been found in a small proportion of autistic people (Dhossche *et al.*, 2002; Schroer *et al.*, 1998). These aberrations include duplications and deletions involving the proximal long arm of this chromosome (Schroer *et al.*, 1998). Three $GABA_A$ receptor subunit genes, GABRB3, GABRA5, and GABRG3, are located in the proximal arm of the 15q region (Schroer *et al.*, 1998). In addition, using fluorescence *in situ* hybridization, Silva *et al.* (2002)

showed tetrasomy in the 15q11–13 chromosomal region in a female child with autism. This tetrasomy could result in an excess of GABA receptors, leading to behavioral problems, including hyperactivity and epilepsy, a frequent comorbidity of autism. Linkage studies for this chromosomal region have produced contradictory results (Bass *et al.*, 2000; Buxbaum *et al.*, 2002; Cook *et al.*, 1998; Maestrini *et al.*, 1999; Martin *et al.*, 2000; Menold *et al.*, 2001; Muhle *et al.*, 2004; Schroer *et al.*, 1998). As previously mentioned, Casanova *et al.* (2002a, 2003) found narrower cell minicolumns in brains from autistics. In this regard it might be of interest that abnormalities in GABAergic interneurons have been suggested to be associated with narrowing of cell minicolumns (Nishikawa *et al.*, 2002). Furthermore, Reelin appears to play a role in the development of minicolumns (Nishikawa *et al.*, 2002), which may imply a relationship between the GABAergic system, minicolumns, Reelin, and autism.

IV. GABA and Brain Development

A. Early Development

Several studies have pointed out that GABAergic synapses and receptors are generated and active before glutamatergic synapses in all brain regions (Ben-Ari *et al.*, 2004; Chen *et al.*, 1995; Koller *et al.*, 1990; Walton *et al.*, 1993). However, GABAergic interneurons have a longer migration journey before they reach their final destination (Ben-Ari *et al.*, 2004). Furthermore, Tyzio *et al.* (1999) have suggested that the first GABAergic synapses are probably located on the apical dendrites of pyramidal neurons and not on the soma.

Many different GABAergic receptor subunits are expressed in the embryonic and/or adult brain. The change in subunit composition is essential for normal development in specific brain regions (Lujan *et al.*, 2005). Each subunit of the $GABA_A$ receptor exhibits a unique regional and temporal expression profile during brain development (Laurie *et al.*, 1992). The $\alpha 2$, $\alpha 3$, and $\alpha 5$ subunits are dominantly expressed during embryonic development whereas others dominate postnatal or adult brain (Fritschy *et al.*, 1994; Laurie *et al.*, 1992). For example, $\alpha 1$ subunit expression is low at birth and increases during the first postnatal week, while the $\alpha 2$ subunit decreases progressively (Fritschy *et al.*, 1994).

During early development, GABA causes depolarization (i.e., excitation) due to a relatively high concentration of intracellular Cl^- (Miles, 1999; Owens *et al.*, 1996). Depolarization occurs via the opening of GABA-gated Cl^- channels, which also increases the intracellular Ca^+ concentration (Jelitai *et al.*, 2004; Miles, 1999; Owens *et al.*, 1996). Both GABA excitation and Ca^+ influx may be important for plasticity, synaptic connections, and for establishing neural

networks (Kriegstein *et al.*, 2001). Consistent with this observation, in primary cultures of several embryonic and neonatal brain tissues, GABA exerts a variety of neurotrophic actions such as promotion of neurite extension, synaptogenesis, and the synthesis of its own receptors (Hansen *et al.*, 1987). Moreover, excitatory GABAergic interneurons can generate giant depolarizing potentials (GDPs), which in turn cause primitive network-driven patterns of electrical activity in all developing circuits (Ben-Ari, 2002). GDPs are hallmarks of developing neural networks and constitute the first synaptic pattern observed in the developing rat hippocampus (between P0 and P10) (Ben-Ari, 2001; Ben-Ari *et al.*, 1989).

During maturation, GABA induces the opposite effect; Cl^- ions are pumped out and the cell becomes hyperpolarized. Thus, GABA switches from an excitatory to an inhibitory neurotransmitter during development (Miles, 1999). In the rat this switch occurs around birth and is due to the expression of the K^+/Cl^- cotransporter 2 (KCC2) (Herlenius *et al.*, 2004; Miles, 1999; Rivera *et al.*, 1999). KCC2 expression increases progressively from embryonic stages to postnatal day 15 (P15) (Stein *et al.*, 2004). This elevation occurs simultaneously with the GABA switch from excitatory to inhibitory (Stein *et al.*, 2004). When the GABA switch occurs in humans is not known (Herlenius *et al.*, 2001).

B. Migration

Migration is a process of neuronal movement essential for the establishment of normal brain architecture. Most cortical neurons migrate from their original site to their final destination in the cerebral cortex using the radial pathway from cortical ventricular zones (VZs) into the neuronal layers. In contrast, a subpopulation of cells including GABAergic neurons move tangentially within the intermediate zone (Hatten, 1999; O'Rourke *et al.*, 1997; Rakic, 1995; Rapp and Bachevalier, 1993). GABAergic neurons arise in the medial and lateral ganglionic eminences and migrate dorsally into the developing cortex (Rapp and Bachevalier, 1993). As early as embryonic day 10 (E10), GABA is located near the target destinations of migratory neurons and in the migrating neurons themselves in the developing mouse brain *in vivo* (Del Rio *et al.*, 2000). Different GABA concentrations promote migration of GABAergic and non-GABAergic neuronal cortical subpopulations (Behar *et al.*, 1996). For example, in cortical regions, femtomolar concentrations of GABA stimulate directed migration (chemotaxis), whereas micromolar levels stimulate chemokinesis (random motility) of more mature neurons (Behar *et al.*, 1996, 1998). Lopez-Bendito *et al.* (2003) showed a modified distribution of tangential migration neurons within the cortex upon blockade of $GABA_B$ receptors with a specific antagonist (CGP52432) in embryonic rat organic cultures. Furthermore, blocking of GABA receptors with saclofen or picrotoxin resulted only in a delay of cell movements, but not a

complete arrest of migration. These findings could indicate that GABA receptor activation in the developing cortex modulates the rate of cell migration rather than initiating it (Behar *et al.*, 2000). Furthermore, it has been suggested that migration, mediated by the GABAergic system, can act through Ca^{2+} ions, which alter cell movements by changing the dynamics of the cytoskeletal remodeling (Gomez *et al.*, 1999)

Other factors regulate tangential migration as well (Marin *et al.*, 2001). Mitogenic factors stimulate the movement of cells, such as hepatocyte growth factor/scattered factor (HGF/SF) and neurotrophic factors. In slice cultures, for example, exogenous HGF/SF increases the number of cells that migrate away from the subpallial telencephalon, while anti-HGF/SF antibodies inhibit cell movement (Powell *et al.*, 2001). Little is known about the substrates that are used by migrating neurons. As described by Parnavelas (2000), it is possible that migrating interneurons use axons as a substrate in the cortex. Nevertheless, it is still unclear whether migrating interneurons interact with fiber tracts *in vivo* (Wichterle *et al.*, 2001). Finally, the factors that guide different migratory streams through appropriate pathways towards their targets (Marin and Rubenstein, 2001), including the netrin/Dcc (Livesey *et al.*, 1997; Parnavelas, 2000), Slit/Robo (Parnavelas, 2000; Yuan *et al.*, 1999), and semaphorin/neuropilin systems (Skaliora *et al.*, 1998).

C. Proliferation

The process of proliferation is responsible for generating the correct number of specific cell types in the correct sequence in the brain (Lujan *et al.*, 2005). Growth factors, neurotransmitters, and their receptors have been implicated in the extrinsic regulation of cell proliferation in the developing telencephalon (Nguyen *et al.*, 2001). In contrast to microglia and astrocytes, neurons exhibit very low rates of proliferation in culture (Eliason *et al.*, 2002). Several studies have demonstrated a positive effect of GABA on cell proliferation. Ben-Yaakov *et al.* (2003) demonstrated GABA-dependent cell proliferation in the hippocampus. GABA also promotes cell proliferation in cultures of cerebellar progenitors, with no effect on cell survival (Fiszman *et al.*, 1999). Furthermore, Haydar *et al.* (2000) showed that exogenous GABA increases proliferation by shortening the cell cycle in the neocortical ventricular zone (VZ) of the embryonic cerebrum in organotypic cultures (Haydar *et al.*, 2000). The reverse effect was found in the subventricular zone (SVZ) (Haydar *et al.*, 2000). The same group demonstrated that the effect seen in the VZ was mediated by $GABA_A$ receptors (Haydar *et al.*, 2000). Activation of $GABA_A$ receptors also influences DNA synthesis (Haydar *et al.*, 2000). However, an *in situ* study demonstrated that $GABA_A$ receptors, triggered by GABA or muscimol (a $GABA_A$ receptor agonist) negatively regulate

DNA synthesis in neural progenitors in the rat embryonic neocortical VZ between E16 and E19 (LoTurco *et al.*, 1995).

In contrast, GABA (and glutamate) has been implicated in the reduction of the number of proliferating cells in dissociated or organotypic cultures of the neocortex (LoTurco *et al.*, 1995). Moreover, Luk and Sadikot (2001) found no GABAergic effect on cell proliferation in the rodent neostriatum. In their study of parvalbumin-immunoreactive progenitors, they only revealed an effect of GABA on cell survival. However, Fiszman *et al.* (1999) demonstrated that GABA and $GABA_A$ receptor agonists do not influence survival in cerebellar granule cells *in vitro*. This finding could be due to a calcium influx via voltage gated calcium channel activation, which subsequently activates the MAPK cascade (Fiszman *et al.*, 1999).

D. Differentiation

Neuronal differentiation is another step in brain development that seems to be regulated by early glutamate- and GABA-mediated signaling. During early cortical neuronal differentiation, Cajal-Retzius (CR) cells play a key role in regulating cortical lamination (Lujan *et al.*, 2005), and they produce Reelin (Nishikawa *et al.*, 2002). Reeler (Reelin mutant) mice display severe cortical laminar disruption (Nishikawa *et al.*, 2002). In addition, CR cells express diverse neurotransmitters and $GABA_A$ receptors, indicating that they can respond to GABA produced by nearby neurons (Mienville *et al.*, 1999). In cultured embryonic hippocampal neurons, $GABA_A$ receptor activation increases neurite outgrowth and maturation of GABAergic interneurons (Barbin *et al.*, 1993). Such activation also plays a role in the morphological development of cortical neurons via membrane depolarization (Maric *et al.*, 2001). In addition, switches in $GABA_A$ receptor unit composition (Owens *et al.*, 1999) and changes in expression of components involved in GABA synthesis, storage, and release may mediate the transition from embryonic to adult GABAergic signaling (Somogyi *et al.*, 1995).

Several factors regulate the differentiation of tangentially migrating neurons, such as Dlx1, Dlx2, and Mash1. These genes may control the timing of neuronal differentiation and induce the GABAergic phenotype. Dlx1 and Dlx2 are linked homeobox genes, whereas Mash1 is a basic helix-loop-helix gene that can induce Dlx1 (Fode *et al.*, 2000). Normally, these three transcription factors are expressed in the VZ and SVZ progenitor cells in the anterior peduncular area (AEP), lateral (LGE), and medial ganglionic eminence (MGE) (Bulfone *et al.*, 1993; Eisenstat *et al.*, 1999; Guillemot *et al.*, 1993; Porteus *et al.*, 1994). Studies with mice lacking Dlx1, Dlx2, or Mash1 have revealed insights in the role of these transcription factors during differentiation. Anderson *et al.* (1997) have shown that the functional loss of Dlx1 and Dlx2 blocks the differentiation of late-born subpallial

telencephalic neurons. Interestingly, double mutations of Dlx1/Dlx2 have a four-fold reduction in GABAergic interneurons (Anderson *et al.*, 2001). Functional loss of Mash1 leads to premature differentiation of several early-born cell populations (Casarosa *et al.*, 1999). In Mash1 mutants, more GABAergic interneurons are lost in the marginal zone of the cortex as compared to the intermediate zone (Marin *et al.*, 2000). Additionally, gain-of-function studies revealed that Dlx and Mash genes both could induce aspects of the GABAergic phenotype (Fode *et al.*, 2000).

V. Animal Models of Autism

Animal models are very useful for determining the role of genes and environment, understanding the pathogenesis, and for testing potential therapeutic approaches. Moreover, animal models need not be perfect mimics of human diseases in order to be valuable. Autism is a heterogeneous disorder in which most of the susceptibility genes have not yet been identified. Nonetheless, there are several genetic changes that do entail a high risk for autism, and mouse models of these changes share some features of the human disorder. Furthermore, models based on environmental risk factors are valuable. Finally, brain lesion models are of interest. The following section describes a variety of animal models that show features of autism.

A. Genetic Manipulation

1. *X-chromosome Loci*

Four loci on the X-chromosome have been identified in autism thus far. These genes are the Fragile X mental retardation protein (Fmr1), methyl-CpG-binding protein type 2 (MECP2), and neuroligin (NLGN) 3 and 4. Fmr1 is silenced in Fragile X syndrome (FXS), a condition that often includes autism symptoms (Wassink *et al.*, 2001). The Fmr1 knockout (KO) mouse displays increased dendritic spine density in the visual and somatosensory cortices, with a greater number of spines with an immature appearance (Comery *et al.*, 1997; Galvez *et al.*, 2003; Nimchinsky *et al.*, 2001). These features have also been found in several cortical areas in FXS humans (Irwin *et al.*, 2001). However, there appears to be a decrease in dendritic branching in the human hippocampus (Raymond *et al.*, 1996). Clearly, more neuropathologic studies are needed to relate this animal model to autism. The NLGN1, 3, and 4 genes map to three loci associated with predisposition to autism, 3q26, Xp22.3, and Xq13, respectively.

Mutations in NLGN3 and 4 are associated with autism and, in some cases, with mental retardation, a feature often associated with autism (Jamain *et al.*, 2003; Laumonnier *et al.*, 2004). Chih *et al.* (2004) and Comoletti *et al.* (2004) found that these NLGN3 and 4 mutations lead to loss of protein processing and loss of the capacity for stimulation of synapse formation. Detailed neuropathology and behavioral analysis of the MECP2 KO remains to be reported.

2. *15q11-q13 Locus*

As previously mentioned, the 15q11-q13 locus is relatively small and has been linked to autism in several studies (Buxbaum *et al.*, 2002; Cook *et al.*, 1998; Dhossche *et al.*, 2002; Menold *et al.*, 2001; Muhle *et al.*, 2004; Schroer *et al.*, 1998). Jiang *et al.* (2004) have identified Ube3a, a genetic locus for Angelman syndrome (AS), which is located in this region and shares some clinical features with autism. The same group investigated a mouse model with a maternal null mutation, which shows a lack of Ube3a expression in cerebellar Purkinje cells and in the hippocampus. Both features have been implicated in autism (Jiang *et al.*, 1998). The relationship of the 15q11-q13 locus and the GABAergic system was previously described. However, more neuropathology is required to establish the relationship between $GABA_A$ receptor KO mice and autism.

3. *Serotonin*

It is generally agreed that there are serotonin (5-HT) abnormalities in autism. 5-HT levels in platelets are increased (Anderson *et al.*, 2002; Cook *et al.*, 1996); however, the underlying mechanism of this elevation is unclear. Therefore, the relevance of these changes for the brain is not well understood. Some studies have emphasized the importance of 5-HT during fetal brain development (Gaspar *et al.*, 2003; Whitaker-Azmitia, 2001), where this transmitter plays a role in neurogenesis, neuronal differentiation, neuropil formation, axon myelination, and synaptogenesis *in vivo* (Whitaker-Azmitia, 2001). Thus, altering 5-HT levels during development could lead to relevant models for autism. Whitaker-Azmitia and colleagues (2001) have investigated 5-HT depleted neonatal rat pups and found decreased dendritic length and spine density in the hippocampus, the same features Raymond *et al.* (1996) observed in autism. Furthermore, neonatal disruption of 5-HT tracts causes alterations in cortical morphogenesis in rodents (Connell *et al.*, 2004).

Studies on KO mice have revealed insights into the role of 5-HT in both early embryonic and postnatal development (Gaspar *et al.*, 2003; Gingrich *et al.*, 2003). For example, KO experiments showed that the $5\text{-}HT_{2B}$ receptor is involved in the regulation of neurogenesis, cell specification, and cell survival during early

development. At later developmental stages, depending on the brain region, such control can be mediated by the 5-HT_{1A} (regulation of dendrite growth) and 5-$HT_{2A/2C}$ (regulation of axonal growth) receptors. Unfortunately, detailed neuropathology in these mice, particularly with relevance to known changes in the autistic brain, is missing.

4. *DLX*

DLX genes regulate the development of a subset of cortical and striatal neurons. Two of the linkage loci for autism, 2q31.1 and 7q21.3, contain the DLX1/2 and DLX 5/6 complexes, respectively. Stuhmer et al. (2002) reported that mutations in DLX2 and 5 genes alter the development of GABA neurons in the forebrain. As stated earlier, the GABAergic system is involved in the pathology of autism. Thus, DLX mutant mice will be of interest.

5. *Engrailed*

The gene *Engrailed 2* (En2) is located on chromosome 7, which has been linked to autism (Gharani *et al.*, 2004). Mouse mutants of En2 and autistic individuals display similar cerebellar morphological abnormalities. En2 KO mice show a loss of Purkinje cells (Gharani *et al.*, 2004; Liu *et al.*, 2001), as do autistic brains, where the loss appears in stripes or patches (Bailey *et al.*, 1998). Other features shared between the En2 KO mice and human autistic cases are deficiencies in the number of deep nuclear, granule, and inferior olive neurons. Recently, increased neuronal packing, a smaller hippocampus, and ectopic location of neuronal subgroups in the amygdala in En2 KO mice have been linked to autism (Kuemerle *et al.*, 1997). There is now a need for detailed behavioral studies of these mice.

6. *Reelin*

The *Reelin* gene, located on chromosome 7, has been linked to autism. Conflicting reports exist about the association of its polymorphisms with autism (Bonora *et al.*, 2003; Krebs *et al.*, 2002; Persico *et al.*, 2001). In the adult brain, Reelin is normally expressed by GABAergic neurons. In patients, Guidotti *et al.* (2000) suggested that there might be a correlation between Reelin and GAD67, one of the two molecular forms of GAD. This interaction is clearly expressed in the cerebellum, where Reelin regulates dendritic sprouting in GABAergic Purkinje cells (Curran *et al.*, 1998). These features were also reported in the heterozygous Reeler mouse (Tueting *et al.*, 1999). Interestingly, in the male heterozygous Reeler mouse (rl+/−), loss of Purkinje cells has been reported between 3 and 16 months of age. Furthermore, mutant Reeler mice, in which Reelin is absent, display severe cortical laminar disruption (Nishikawa *et al.*, 2002).

B. Environmental Factors

1. *Thalidomide and Valproic Acid*

Thalidomide has been associated with a marked increase in the incidence of autism (Stromland *et al.*, 1994). In rats, thalidomide exposure on E9 causes increased plasma, hippocampal, and frontal cortex 5-HT as well as an altered distribution of 5-HT in neurons in the raphe nuclei (Narita *et al.*, 2002). The offspring of women taking valproic acid (VPA) during early pregnancy have an increased risk for autism (Costa *et al.*, 2004). The offspring of pregnant rats given VPA show a reduced number of Purkinje cells, decreased cerebellar volume, and a decreased cell number in the cranial nerve motor nuclei (Ingram *et al.*, 2000). In addition, neurons in the inferior olive that innervate Purkinje cells are also reduced in number, as are those in the deep nuclei targets of Purkinje cells in the nucleus interpositus (Rodier *et al.*, 1996). As with thalidomide, VPA exposure on E9 causes hyperserotonemia in the mouse hippocampus, frontal cortex, and cerebellum (Narita *et al.*, 2002). These observations all parallel human autistic pathologic findings. The use of thalidomide and VPA has provided important insights into autism and has led to useful animal models as well.

2. *Maternal Infection*

Maternal infection increases the risk of autism in the offspring. For example, prenatal exposure to rubella virus increases the incidence of autism (Chess, 1977). Furthermore, pregnant mice infected with influenza virus at E9.5 yield adult offspring that display histological and behavioral abnormalities found in autism and schizophrenia (Shi *et al.*, 2003). These mice have smaller brain sizes at birth, but macroencephaly in adulthood (Fatemi *et al.*, 2002a; Shi *et al.*, 2003). This neonatal undergrowth followed by overgrowth mirrors the pattern seen in autism. With respect to brain pathology, the offspring of maternally infected mice display a selective loss of Purkinje cells in lobule VII, as well as thinning of the neocortex and hippocampus, pyramidal cell atrophy, reduced levels of Reelin immunoreactivity, and changes in neuronal nitric oxide synthase expression and synaptosome associated protein of 25kDa (SNAP-25) (Fatemi *et al.*, 1998, 2000a, 2002a; Shi *et al.*, 2003). Some of these features mimic autism pathology.

3. *Postnatal Viral Infection*

The most extensively studied rodent model involving postnatal viral infection utilizes intracerebral injection of Borna disease virus (BDV) within 12 hrs of birth (Hornig *et al.*, 2003; Pletnikov *et al.*, 2002). Several aspects of this model are relevant to autism, including alterations in 5-HT levels in various brain regions, loss of Purkinje cells in the cerebellum, and granule cells in the dentate gyrus (Dietz *et al.*, 2004). Postnatal infection with lymphocytic choriomeningitis virus

(LCMV) also leads to acute loss of neurons in the cerebellum and delayed loss of neurons in the hippocampus (Pearce, 2003). There is also an association between cytomegalovirus (CMV) infection and autism, which could be followed up in animals (Yamashita *et al.*, 2003).

C. Lesions

1. *Cerebellum*

Neuropathologic observations in autism are rather consistent in respect to the cerebellum. Therefore, it is of particular interest to study genetic, surgical, and toxin lesions of the cerebellum. Recently, numerous mutations and toxic insults were associated with diverse patterns of Purkinje cell loss (Sarna and Hawkes, 2003). Subpopulations of Purkinje cells are less vulnerable to death, probably because they express the neuroprotective protein HSP25/27. The same feature is seen in mouse models of Niemann-Pick disease type A/B and C (Sarna and Hawkes, 2003). Accordingly, the pattern of cell loss in each case is specific to the type of insult. Thus, the selective loss of Purkinje cells in lobule VII in the influenza model is an important parallel with autism.

The various models discussed here are the primary ones showing neuropathologies that mimic some of the features found in autism. However, striking behavioral features of autism can be assayed in animals, such as stereotypic and repetitive behaviors, enhanced anxiety, abnormal pain sensitivity, disturbed sleep patterns, deficient maternal bonding/affiliation, and deficits in sensorimotor gating (prepulse inhibition, PPI). While the maternal infection, thalidomide, and VPA models have been studied behaviorally, much remains to be done in this respect with the other models.

VI. Discussion

In this chapter we have examined the literature on autism neuropathology, the role of the GABAergic system in this disorder, and the relevance of mouse models showing features of autism. With respect to the neuropathology of autism, consistent findings have emerged for the limbic system, cerebellum, and cerebral cortex. Neuropathologic data of the limbic system show increased cell packing density and smaller neurons. These observations might be explained by an arrest of normal development (Kemper and Bauman, 1998). A decreased number of cerebellar Purkinje cells without significant gliosis and features of cortical dysgenesis have been reported by several different research groups. These findings

suggest a largely prenatal origin of autism, which is supported by the epidemiological findings with thalidomide, VPA, and maternal infection. Furthermore, age-related abnormalities in the inferior olive and cerebellar nuclei have been found in autism. As the inferior olive project to the cerebellar Purkinje cells, a decrease in Purkinje cell number can be caused by a very early, abnormal development of these projections (Palmen *et al.*, 2004). Cortical dysgenesis might affect correct lamination of the cortex and can cause abnormalities in the process of cell death. In line with this are findings of reduced Reelin levels and Bcl-2. Reelin also plays a role in the development of minicolumns, a feature that has been reported in autism. Furthermore, abnormalities in GABAergic neurons are associated with narrowing of the cell minicolumns. However, those studies involve small sample sizes, the use of quantification techniques not free from bias, and high percentages of autistic subjects with comorbid mental retardation or epilepsy.

As with the cholinergic system, several studies have reported a reduction in GABA function, availability, and activity in autism. Furthermore, a decrease in GABA receptor binding has been shown in autism. An imbalance in the availability of GABA receptors subunits may alter receptor activity and hence change the activity of the brain's major inhibitory neurotransmitter (Schroer *et al.*, 1998). As a consequence, the threshold for developing seizures, a frequent comorbidity of autism, might be reduced. From a genetic point of view, three $GABA_A$ receptor subunit genes have been located in the proximal arm of chromosome 15. Abnormalities in this chromosomal region have been found in a small proportion of autistic people.

As autism is a neurodevelopmental disorder, it is important to elucidate the role of GABA during development. Evidence indicates that GABA plays a role in several developmental processes including cell migration, proliferation, and differentiation. As these processes might be affected in autism, GABA dysfunction could account for some neuropathology seen in autism. For example, GABA dysfunction may occur in conjunction with Reelin and therefore affect neuronal migration and the arrangement of cortical minicolumns in autism. The results reported on the influence of GABA on proliferation are somewhat controversial. A positive influence on GABA cell survival could reflect a GABA mediated decrease in apoptosis during development and in turn might explain the increased head circumference, brain weight, and brain volume reported in autism. However, when abundant cells are not functioning properly, they would die, resulting in normalization of, or a decrease in, brain volume later in childhood. However, in those subgroups of autistic subjects in which this brain enlargement is still present in adulthood, this compensatory cell death may not take place (Courchesne, 2004). Instead, these autistic subjects might be able to recruit these 'extra' neurons, possibly resulting in increased dendritic growth and thus in increased brain volume, still present in adolescence.

Although the neuropathologic results in autistic subjects are revealing, animal models are essential for better understanding of the pathophysiology, cause, and treatment of autism. Although no animal model has been created that perfectly mimics all aspects of a human disease, striking parallels between autism and animal models have already been reported. For example, in genetic models, mouse mutants of En2 display decreased Purkinje cell number (Gharani *et al.*, 2004). In NLGN mouse mutants, a loss of protein processing and a loss of the capacity for synapse stimulation are found (Chih *et al.*, 2004). DLX mutations are associated with abnormalities in the development of GABAergic neurons (Stuhmer *et al.*, 2002). The recent linkages of NLGN, DLX, and En2 to autism offer possibilities for animal models, particularly if introducing the relevant, specific mutations (as opposed to simple KOs) can cause interesting pathology and behavior. In addition, infection with Borna disease virus shows behavioral disturbances in sensorimotor, emotional, and social activity, together with a decrease in the number of Purkinje cells (Hornig *et al.*, 2002). Maternal infection models display selective Purkinje cell loss (Patterson, 2002; Shi *et al.*, 2003). This model also shows macroencephaly in the offspring, atrophy of pyramidal cells, and reduced Reelin immunoreactivity. Furthermore, these mice display deficits in social behavior, social interaction, and PPI.

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THE ROLE OF GABA IN THE EARLY NEURONAL DEVELOPMENT

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A large body of experimental evidences demonstrates that GABA signaling possess an inherent capability for potent regulation of almost all steps of neuronal differentiation and neural tissue formation. The transient GABA production is an inherent feature of many differentiating neuronal populations, regardless of the future neurotransmitter phenotype.

While in the mature CNS GABA acts mainly as a synaptic neurotransmitter and elicits phasic responses, in the developing nervous tissue GABA acts as an autocrine/paracrine signal molecule and its main roles are executed through tonic signaling. In young differentiating neurons, most important effects of GABA are mediated through $GABA_A$ receptors and result in membrane depolarization, and in an increased $[Ca^{2+}]_I$, which in turn can stimulate multiple cellular processes. $GABA_B$ receptor-mediated effects, which can cause hyperpolarization and reduce $[Ca^{2+}]_I$, appear in later phases of tissue genesis, and seem to balance $GABA_A$ signaling.

The effects of GABA had been demonstrated in the entire period of neural tissue genesis, from proliferation, through migration and differentiation of

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neuronal precursors, up to the synapse formation and circuit refinement by maturing neurons. The most intriguing developmental role that has been attributed to GABA is the generation and maintenance of activity waves in the period of functional network formation.

I. Introduction

Nearly all organisms contain and synthesize γ-aminobutyric acid (GABA), from bacteria (Ackermann, 1910) through plants (Steward *et al.*, 1949) up to humans (Elliott and Jasper, 1959). In plants, GABA serves as an important metabolic compound, but its role as a signal molecule was also demonstrated (Bouche *et al.*, 2003). The presence of GABA and the similarity of GABA signaling in a broad range of tissues and organisms argue for the importance of phylogenetically conserved GABA-functions.

In the mammalian brain, GABA was recognized more than 50 years ago (Awapara *et al.*, 1950). Soon after, strong evidence indicated that GABA acts as an inhibitory neurotransmitter in both the vertebrate and invertebrate nervous system. Beside the accumulation at presynapses and in the postsynaptic densities, different types of GABA receptors were shown in extrasynaptic locations on various types of neurons (Farrant and Nusser, 2005) and also on non-neuronal cells (Watanabe *et al.*, 2002). During the last 20 years, it was revealed that most neuronal precursors respond to GABA by depolarization in defined phases of differentiation. GABA-induced anion fluxes result in hyperpolarization in later stages of development, in conjunction with the maturation of neuronal ion-homeostasis (Owens and Kriegstein, 2002). In the developing mammalian central nervous system, many neurons and glial cells produce and release GABA. Besides its excitatory or inhibitory neurotransmitter functions, GABA serves also as an autocrine/paracrine signal molecule, and plays important regulatory roles in the entire period of neural development.

In this chapter we give a summary of the recent knowledge of the development of GABA signaling and of the GABA actions, which contribute to the formation of the neural tissue. The first part describes the developmental changes in the composition and distribution of the GABA signaling system. In the second part, we intend to summarize the recent understanding of GABA effects on distinct steps of neuronal differentiation. The chapter does not focus on glial development, and the important modulatory roles of glial cells in almost all aspects of GABA signaling are only occasionally mentioned.

II. The GABA Signaling System in the Developing CNS

Components of the GABA signaling system, GABA and GABA synthesizing enzymes, GABA receptors, GABA transporters, and GABA metabolizing mechanisms appear early in development (Barker *et al.*, 1998). In many areas of the developing nervous system, the components of GABA signaling machinery show transient overexpression.

A. The Presence of GABA in the Developing Brain Tissue

In the extracellular environment of the adult brain, GABA is present in concentrations between 0.5–1 μM (Lerma *et al.*, 1986). In the surroundings of adult progenitors in the subventricular zone, however, elevated (2–3 μM) GABA concentrations were reported (Bolteus *et al.*, 2005). In immature human brain, the GABA content in the cerebro-spinal fluid exceeds the adult level (Hedner *et al.*, 1982). The environment of developing neural cells is enriched in GABA (Benitez-Diaz *et al.*, 2003).

The elevated GABA-level in the developing neural tissue is the result, in part, of immature functioning of the blood brain barrier (Engelhardt, 2003). In neonates, enhanced transport-rate of GABA was demonstrated from the choroid plexus to the CSF (Al-Sarraf, 2002).

An important shift in the balance between GABA release and uptake has been shown as another source to enhance the extracellular GABA concentration in the developing neural tissue. Many populations of developing neuronal precursors produce and release GABA in defined stages of differentiation. *In vitro* studies demonstrated a continuous GABA-release from developing hippocampal cells (Valeyev *et al.*, 1993) and revealed a continuously elevated (3–10 μM) GABA concentration in the environment of neural stem cells (Jelitai *et al.*, 2004). Immunocytochemical studies on young embryos showed that GABA-containing cells and fibers are present, well before the onset of synaptogenesis (see Box 1). In the period of the formation of the cortical plate, the early developing Cajal-Retzius cells (Marin-Padilla, 1998) and cortico-petal fibre tracts contain GABA (Lauder *et al.*, 1986). Sparse GABA-containing cells were also detected in the developing rat striatum as early as E13 (Fiszman *et al.*, 1993). The number of GABA-positive cells increased, in parallel with the immigration of future interneurons derived from the subventricular zone (Lavdas *et al.*, 1999). By E16, many layers of the developing rat forebrain cortex contain GABA-positive cells (Lauder *et al.*, 1986). GABA-containing cells were also demonstrated in the human neocortex in early periods of its organization (Zecevic and Milosevic, 1997). Besides some transient "organizer" and future GABAergic neurons (Box 1), many precursors also seem to produce and release GABA, transiently (Verney, 2003).

Box 1

The origin and development of GABAergic neurons is best understood in the development of the cerebral cortex. As a first sign of cortical development, Cajal-Retzius cells are migrating from the ventricular zone to the marginal zone along radial glial cells (Rakic, 1972). These cells produce reelin (D'Arcangelo *et al.*, 1995) and GABA and play an important role in directing the arrangement of later arriving neuronal precursors.

The majority of cortical GABAergic interneuron precursors are generated in the basal ganglia anlage (Anderson *et al.*, 1999), in the medial (MGE) lateral (LGE), caudal (CGE), and retro-bulbar ganglionic eminences (Xu *et al.*, 2004), and migrate to their cortical destination by taking tangential and radial routes (Kriegstein and Noctor, 2004). The precise origin and migratory paths of GABAergic interneuron populations may differ in different species. In humans, more than half of the cortical interneurons were reported to derive from the cortical subventricular zone (Letinic *et al.*, 2002).

GABA is distributed diffusely throughout the cytoplasm of immature neuronal precursors, as vesicular GABA storage mechanisms develop relatively late (Minelli *et al.*, 2003a). Spontaneous GABA release was shown from cell bodies, growth cones, and along the neurites of developing neurons (Balcar *et al.*, 1983). The mechanism of spontaneous GABA release, however, is not properly understood. *In vitro* (Taylor *et al.*, 1990) and *in vivo* (Demarque *et al.*, 2002) evidences indicate that there is an early, Ca-independent GABA release, which can be provoked by K^+-influx, and so, presumably, by depolarization.

Later, in the periods of neuronal network formation, GABA is secreted—at least in part—through reverse action of transporters (Attwell *et al.*, 1993), which can be stimulated by mild depolarization (Belhage *et al.*, 1993).

Specific GABA transporters (GATs) (see Box 2) have a pivotal role in the maintenance of extracellular GABA concentration. In adults, GATs can terminate GABA signals by reuptake of GABA into nerve terminals and into the surrounding glial cells. In early phases of the neural tissue development, however, GABA-uptake seems to underfunction (Demarque *et al.*, 2002).

B. GABA Transporters

GABA transporters display characteristic cellular distribution and regional expression patterns (Borden, 1996). To date, four GABA transporters, GAT1-3 and BGT-1 (in nomenclature used for mouse GAT1-4) (Liu *et al.*, 1993) have been cloned (Guastella *et al.*, 1990).

Box 2

The cerebral cortex contains GAT-1 at the highest level, and GAT-3, GAT-2, and BGT-1 in decreasing amount, respectively. GAT-1 and GAT-3 are expressed exclusively in the brain. The various GATs display different ion-dependency and pharmacological properties (Gadea and Lopez-Colome, 2001). The transporters can undergo rapid redistribution between surface and intracellular compartments, and their function can be regulated by phosphorylation (Hansra *et al.*, 2004) and by intermolecular interactions (Quick *et al.*, 2004).

In mature GABAergic neurons, a distinct carrier, the vesicular GABA transporter (VGAT), sorts GABA in at least two separate pools: the cytoplasmic and the vesicular pool. For synaptic release, GABA is loaded into synaptic vesicles by VGAT and then liberated from nerve terminals by calcium dependent exocytosis.

GAT-1 was demonstrated both in neurons and astrocytes. During synaptogenesis, it is transiently overexpressed in both neuronal and astrocytic cell bodies (Xia *et al.*, 1993). In the marginal zone of developing cerebral cortex, GAT-1 mRNA appears at late embryonic stages in both humans (Hachiya and Takashima, 2001) and rats (Jursky and Nelson, 1996). The expression is weak in the neonatal cortex, and increases gradually to the mature pattern reached after one month of birth in rodents (Jursky and Nelson, 1996). Efficient GABA uptake through functional GAT-1 was demonstrated only in postnatal periods (Sabau *et al.*, 1999). In the developing cortex, however, postnatal GABA uptake transiently exceeds the adult level (Coyle and Yamamura, 1976).

The expression of GAT-2 is more pronounced in the developing than in the mature CNS (Liu *et al.*, 1993). GAT-2 containing astrocytic processes is more abundant around blood vessels in the neonatal than in adult cortex (Ikegaki *et al.*, 1994). In adults, GAT-2 was detected in epithelial and glial cells and also in neurons (Conti *et al.*, 1999). GAT-2 is expressed in the pial meninges and along the arachnoid trabeculae. GAT-2 seems to regulate the GABA level in the CSF and the GABA transport across the blood-brain barrier in the developing CNS.

GAT-3 is expressed mainly by glial cells, but it was demonstrated in perinatal cortical GABAergic neurons, as well (Durkin *et al.*, 1995). In late embryonic stages, GAT-3 expression has been demonstrated in the marginal and intermediate zones of the cerebral cortex (Evans *et al.*, 1996). In neonates, GAT-3 is present throughout the cortical wall (Jursky and Nelson, 1999). In contrast to GAT-1, GABA-uptake through GAT-3 could be demonstrated in the periods of fetal cortico-genesis (Conti *et al.*, 2004). GAT-3, together with GAT-2, was detected also around the cortical blood vessels (Minelli *et al.*, 2003b) in neonates. The presence of functional GAT-3 in regions of migrating neuronal precursors

indicates that this GABA-transporter plays a role in the regulation of the tonic GABA-level in the early periods of neural tissue genesis. The functional importance of GATs and the onset of GABA uptake in various regions of developing CNS, however, have not been fully elucidated, yet.

C. Synthesis of GABA: GADs

GABA is synthesized primarily from glutamate in a reaction that is catalysed by two glutamic acid decarboxylase (GAD) enzymes: GAD65 and GAD67 coded by distinct genes (Erlander *et al.*, 1991). The two forms seem to synthesize GABA for specific pools, which serve distinct functions (Soghomonian and Martin, 1998). The GAD67 isoform is diffusely distributed in the cytoplasm and produces GABA mainly for the metabolic pool and for extrasynaptic release (Kanaani *et al.*, 1999). The majority of the GAD65 enzyme isoforms are associated with membranes and are enriched in the neuronal terminals (Martin, 2004). The association with synaptic vesicles indicates that GAD65 is involved in the synthesis of vesicular GABA, which mediates fast synaptic communication (Hsu *et al.*, 2000).

GAD enzymes were detected as early as the ninth day after conception in non-neural regions of mouse embryos (Maddox and Condie, 2001). During embryonic development, different GAD67 transcripts, truncated the "embryonic" (25-kDa and 44-kDa) GAD proteins are produced (Bond *et al.*, 1990). The enzymatically inactive GAD25 is widespread at earlier developmental stages (from E10.5-E12.5 in mouse) (Szabo *et al.*, 1994), in regions characterized by proliferation and initial differentiation of future neurons. There are postmitotic neuronal precursors in the embryonic (E13) rat spinal cord, which are GAD+ but GABA− (Ma *et al.*, 1992) and express enzymatically inactive GAD25 (Behar *et al.*, 1993). GAD44, the enzymatically active embryonic form, was detected in mouse CNS in a period from E11 to P21 (Szabo *et al.*, 1994). Embryonic forms of GAD 67 are transiently expressed also in the developing telencephalon, mainly at sites with active neurogenesis and containing migrating or postmigratory neuronal precursors (Behar *et al.*, 1994). The adult form of GAD67 appears around mid-gestation, rises rapidly until birth, and reaches the adult level in mouse 4 weeks after birth (Szabo *et al.*, 1994). With the expression of the full-length form of GAD67, the percentage of GABA+ cells increases (Behar *et al.*, 1993). The sequential appearance of the individual GAD forms clearly indicates that they perform different developmental functions. The widespread though transient appearance of GAD44 suggests that this embryonic GAD protein might contribute to transient GABA production, while for the 25 kDa form, some distinct regulatory functions can be postulated (Varju *et al.*, 2002).

D. Metabolic Removal of GABA

The fraction of GABA taken up by neurons is mainly recycled into synaptic vesicles, and used repeatedly for synaptic communication. In astrocytes, a large part of GABA is metabolized via the citrate cycle. Through this pathway, GABA can serve as an intermediary metabolic compound (Waagepetersen *et al.*, 1999). GABA is transformed to succinic semialdehyde by transamination reaction catalysed by the GABA transaminase (GABA-T) enzyme (Balazs *et al.*, 1970). GABA-T is associated with the inner mitochondrial membrane (Salganicoff and Derobertis, 1965) and its activity plays an important role in setting the GABA-level of the local tissue-environment. Therapeutical modification of GABA-T activity provides a possible tool for setting extracellular GABA-concentration in various psychiatric and neurological disorders (Sarup *et al.*, 2003).

E. The GABA Receptors

Receptors for GABA are divided into two main classes: $GABA_A$ receptors, which are members of the ligand-gated ion channel superfamily and $GABA_B$ metabotropic receptors which belong to G protein-linked receptors. Both types of GABA receptors are abundant in the brain, and found in almost all types of neurons and many populations of glial cells. On neurons, $GABA_A$ and $GABA_B$ receptors can be situated at both synaptic and extrasynaptic sites.

1. *The Structure and Pharmacology of $GABA_A$ Receptor*

In response to GABA binding, the $GABA_A$ receptor-channel opens up, and anions flow inward or outward through the channel-pore, depending on the electrochemical gradients of the permeant ions. $GABA_A$ receptors carry primarily chloride ions but bicarbonate can also permeate, although less efficiently (Bormann *et al.*, 1987).

$GABA_A$ receptors are heteropentameric complexes of subunit proteins coded by several related genes (Macdonald and Olsen, 1994). At present, $\alpha(1–6)$, $\beta(1–3)$, $\gamma(1–3)$, $\varepsilon(1)$, $\pi(1)$, $\theta(1)$, $\rho(1–3)$, and $\delta(1)$ subunits have been identified, and some further variations are produced by alternative splicing. This multiplicity provides a large variety of potential subunit-combinations. However, there are certain preferred subunit combinations, as not all subunits can efficiently co-assemble (McKernan and Whiting, 1996). The variety is further limited by the availability of the subunits, which is restricted by the temporal and spatial pattern of subunit expression (Owens and Kriegstein, 2002).

Depending on the subunit composition, receptors with distinct electrophysiological properties will emerge, which may display also different ligand binding and gating properties (Bohme *et al.*, 2004). The cellular distribution and turn over

of the receptors are also influenced by the subunit composition (Luscher and Keller, 2004). The characteristic spatial-temporal patterns of the subunit expression well correlates with the qualitative differences observed in the properties of $GABA_A$ receptors (Owens *et al.*, 1999) (see Box 3).

Box 3

GABA(A) receptor functions are further modulated by a variety of compounds, among them a number of clinically important drugs, such as benzodiazepines, barbiturates, steroids, anesthetics, and convulsants (Macdonald and Olsen, 1994). Many clinically relevant effects occur through interactions with distinct allosteric binding sites on $GABA_A$ receptors. The effects of these chemicals depend on the subunit composition of the receptors. Synaptic and extrasynaptic receptors are composed by different subunits and, accordingly, display different sensitivity to several drugs. Differences in the subunit composition are reflected by the non-equal effects of benzodiazepine site binding ligands on the phasic and tonic actions of GABA (Farrant and Nusser, 2005).

Generally, two α, two β, and a γ subunits produce the native GABA(A) receptors. The α and β subunits are responsible for ligand binding, while γ δ, ε, or π subunits play important roles in membrane-positioning of the receptors. The γ subunits were shown to be responsible for the accumulation of the receptors at the active postsynaptic sites (Essrich *et al.*, 1998). Receptors containing a $\gamma 2$ subunit in association with $\alpha 1$, $\alpha 2$, or $\alpha 3$ subunits ($\alpha 1 \beta 2/3$ $\gamma 2$, $\alpha 2 \beta 2/3$ $\gamma 2$, or $\alpha 3 \beta 2/3$ $\gamma 2$) are the predominant receptor subtypes in the mature postsynaptic densities (Farrant and Nusser, 2005). It seems that in some receptor complexes the δ, ε, or π subunits can replace the γ, whereas the θ subunit can stand instead of a β subunit (Sieghart *et al.*, 1999). ρ subunits can form either homo- or heteromeric channels, which display special properties. These channels are insensitive for bicuculline and were previously thought to compose a distinct type of receptors, called $GABA_C$ (Barnard *et al.*, 1998).

2. *The Expression of $GABA_A$ Receptors During Development*

$GABA_A$ receptor mediated responses can be recorded from the very early stages of neuronal development. With the advancement of neural differentiation, the subunit-composition and the cellular distribution of $GABA_A$ receptors, as well as the ionic homeostasis of the cells, are changing. As a consequence, $GABA_A$ mediated responses will characteristically change in the course of development.

Expression of different $GABA_A$ receptor subunits shows a unique regional and temporal profile during development (Box 4). In the rat forebrain, $\alpha 4$, $\beta 1$, $\gamma 1$ subunit proteins are present in proliferating neuroepithelial cells in the

ventricular zone (Ma and Barker, 1995). During migration from the germinative zone to the cortical plate, the subunit pattern changes and an $\alpha 3$, $\beta 2/3$, $\gamma 3$ combination becomes dominant (Maric *et al.*, 2001). It seems that there is a shift in the subunit expression when the cells change from the proliferative to a postmitotic stage. In the fetal nervous system (E17–20 in rat), the $\alpha 5$, $\beta 2/3$, and $\gamma 2$ subunits were distributed almost uniformly, while the expression of $\alpha 2$, $\alpha 3$ varied markedly among various regions (Serafini *et al.*, 1998). It seems, that in young differentiating neurons, most $GABA_A$ receptors are composed of combinations of $\alpha 2/3/5$-, $\beta 2/3$-, and $\gamma 2/3$ (Ma and Barker, 1995). Some of the subunits (as $\alpha 5$, $\gamma 1$ and $\gamma 3$) are transiently expressed at a higher level in the course of embryonic and early postnatal development.

Box 4

$\alpha 4$, $\beta 1$, $\gamma 1$ transcripts are expressed in the ventricular zone, but they have been detected in zones of postmitotic differentiating neurons, as well. δ subunits, which are expressed mainly postnatally and at extrasynaptic sites, have been described at low levels also in the cortical plate (Laurie *et al.*, 1992). $\alpha 5$ transcripts were detected in the embryonic brain, with a prominent peak in early postnatal development and a marked decreased afterwards (Poulter and Brown, 1999). The $\alpha 6$ subunits are expressed predominantly in cerebellum, and mainly extrasynaptically (Mody, 2001). Among the γ subunits the $\gamma 1$ and $\gamma 3$ gene expression level decreased during development, while $\gamma 2$ remained mostly constant (Laurie *et al.*, 1992).

Neurotransmitter spillover and non-synaptic release are thought to underlie the activation of extrasynaptic receptors in adults (Semyanov *et al.*, 2004). Continuous activation of extrasynaptic $GABA_A$ causes tonic signaling (Mody, 2001). The endogenous GABA level (0.5–3 μM) is sufficiently high to generate tonic conductance in certain regions (such as cerebellum and hippocampus) of the adult brain (Kaneda *et al.*, 1995). The receptors that mediate tonic inhibition have higher affinity for GABA than the receptors mediating phasic inhibition (Stell and Mody, 2002). δ subunit-containing receptors are present exclusively at extrasynaptic membranes (Nusser *et al.*, 1998). The contribution of δ subunit results in non-desensitization upon prolonged activation and 50-fold higher affinities for GABA (Saxena and Macdonald, 1994). The $\alpha 4$ subunit was found mostly extrasynaptically in hippocampal granule cells (Sun *et al.*, 2004). The $\alpha 5$ was suggested as a non-aggregating, extrasynaptic subunit, because it was homogenously distributed over the cell surfaces (Sassoe-Pognetto *et al.*, 2000).

During postnatal development, the receptors containing $\alpha 2$ subunits are gradually replaced by receptors containing $\alpha 1$ subunits (Fritschy *et al.*, 1994). The increased $\alpha 1$ participation in the receptors is responsible for the fast inhibitory synaptic currents that are normally observed from the postnatal periods of development (Vicini *et al.*, 2001).

In many regions of the developing CNS, the spatial distribution of $GABA_A$ receptors does not follow strictly the potential GABA-sources (e.g., the distribution of GABA-containing cells). As an example, $GABA_A$ receptors were detected in the germinative layer of fetal rats, while GABA-containing neurons or neurites were demonstrated in more superficial layers in the developing forebrain (Cobas *et al.*, 1991). Such "mismatch" indicates the paracrine action of GABA, what is expected in periods preceding synaptogenesis.

In contrast to trans-synaptic, neurocrine signaling, paracrine GABA receptors are not accumulated into dense patches, but are roughly evenly distributed along extended areas of the cell surfaces. Detection of GABA at low paracrine-range (10^{-5}–10^{-7}M) requires high affinity and non-desensitizing receptors. During early embryonic development, $GABA_A$ receptors have higher affinity to GABA (Hevers and Luddens, 1998) and slower decay kinetics than the postsynaptic receptors (Hollrigel and Soltesz, 1997). In many respects, these early paracrine receptors are similar to receptors working in the extrasynaptic membranes of mature neurons.

3. *Excitation Instead of Inhibition*

In the early phases of neuronal development, $GABA_A$ receptor activation causes depolarization in the cells instead of hyperpolarization. In immature neurons, due to a relatively high intracellular Cl^- concentration, the resting membrane potential is more negative than the reversal potential of chloride (Cherubini *et al.*, 1991). Upon opening of GABA-gated Cl^- channels, Cl^- will diffuse out of the cells. The high chloride concentration is due to the function of sodium- and potassium-coupled co-transporter (NKCC1) and the lack of potassium-chloride co-transporter (KCC2) (Clayton *et al.*, 1998). Thus, the opening of GABA-gated chloride channels results in a net chloride outflow and depolarization. Through depolarization, and via voltage sensitive L-type calcium channels, the activation of $GABA_A$ receptors increases the cytosolic $[Ca^{2+}]$ (Yuste and Katz, 1991). As development proceeds, neuronal $[Cl^-]_I$ decreases in conjunction with the onset of the function of KCC2, the Cl^--extruding neuronal transporter (Rivera *et al.*, 1999). Therefore, the $GABA_A$ reversal potential becomes more negative, and Cl^- will flow into the cells through open anion channels. This shift will result in the establishment of the hyperpolarizing GABA effect (Owens *et al.*, 1996).

F. The $GABA_B$ Receptors

$GABA_B$ receptors were described in the early 1980s (Bowery *et al.*, 1980), when chloride-independent and bicuculline insensitive GABA responses were found. The GABA-mediated responses through these receptors were antagonized

by baclofen. Through G protein coupled reactions, the activation of $GABA_B$ receptors can result in a decrease of cAMP level and in the modification of various ion channels (Fig. 1) (Bettler *et al.*, 2004). Signaling through $GABA_B$ receptors can reduce the presynaptic neurotransmitter release by inhibiting voltage dependent Ca^{2+} channels and decreasing $[Ca^{2+}]_I$. Activation of postsynaptic $GABA_B$ receptors activates inwardly rectifying potassium channels (GIRK) and can hyperpolarize the postsynaptic membrane (Luscher *et al.*, 1997) depending on the type of GIRKs (Cruz *et al.*, 2004). Novel data indicate that $GABA_B$ receptors also can act as calcium-sensors (Galvez *et al.*, 2000). On Purkinje cells, they can associate with mGluR1 metabotropic glutamate receptors and enhance neuronal metabotropic glutamate signaling on an outside Ca-level dependent, but G protein independent way and in the absence of GABA (Tabata *et al.*, 2004).

The $GABA_B$ receptors occur both pre- and postsynaptically, but are located mostly at extrasynaptic sites (Bettler *et al.*, 2004). The dominant extrasynaptic localization suggests that the $GABA_B$ receptor signaling mediates mainly non-synaptic GABA effects. In the developing nervous tissue, $GABA_B$ activation seems to counteract to $GABA_A$ signaling and reduces or prevents the depolarization and Ca-increase elicited by GABA through $GABA_A$ receptors. The presence of both types of receptors on the same cell provides a sensitive control of GABA action depending on the composition of the receptors and on the extracellular GABA-level.

1. *The Structure of $GABA_B$ Receptors*

The main $GABA_B$ receptor subunits (termed $GABA_BR1$ and R2 and $GABA_{BL}$) are encoded by distinct genes (Nakayasu *et al.*, 1993). From the $GABA_B1$ gene several isoforms can be translated (referred as 1a-1g), and splice variants of $GABA_{B2}$ have been also described (Bettler *et al.*, 2004). In the brain, $GABA_{B1a}$ and $GABA_{B1b}$ are the most abundant $GABA_{B1}$ isoforms in many species including human (Kaupmann *et al.*, 1998b). The various subunits provide functional receptor heterogeneity, with different pharmacological sensitivities and preferential subcellular locations.

The functional receptors contain two different subunits (Fig. 1). The $GABA_{B1}$ subunit is essential for ligand binding, whereas the $GABA_{B2}$ subunit is required for G-protein coupling and for efficient trafficking to the cell membrane (Kaupmann *et al.*, 1998a) (Box 5). The $GABA_{BL}$ can not form functional receptors with either $GABA_{B1}$ or $GABA_{B2}$ (Calver *et al.*, 2003).

Results obtained from studies on knockout mice demonstrated that $GABA_{B1}$ subunits are needed for all $GABA_B$ receptor-mediated responses in the CNS (Schuler *et al.*, 2001). Mice lacking $GABA_{B2}$ subunits, on the other hand, exhibit epileptiform seizures and behavioral symptoms reminiscent of $GABA_{B1}$ knockout mice (Thuault *et al.*, 2004) indicating the necessity of both subunits for normal

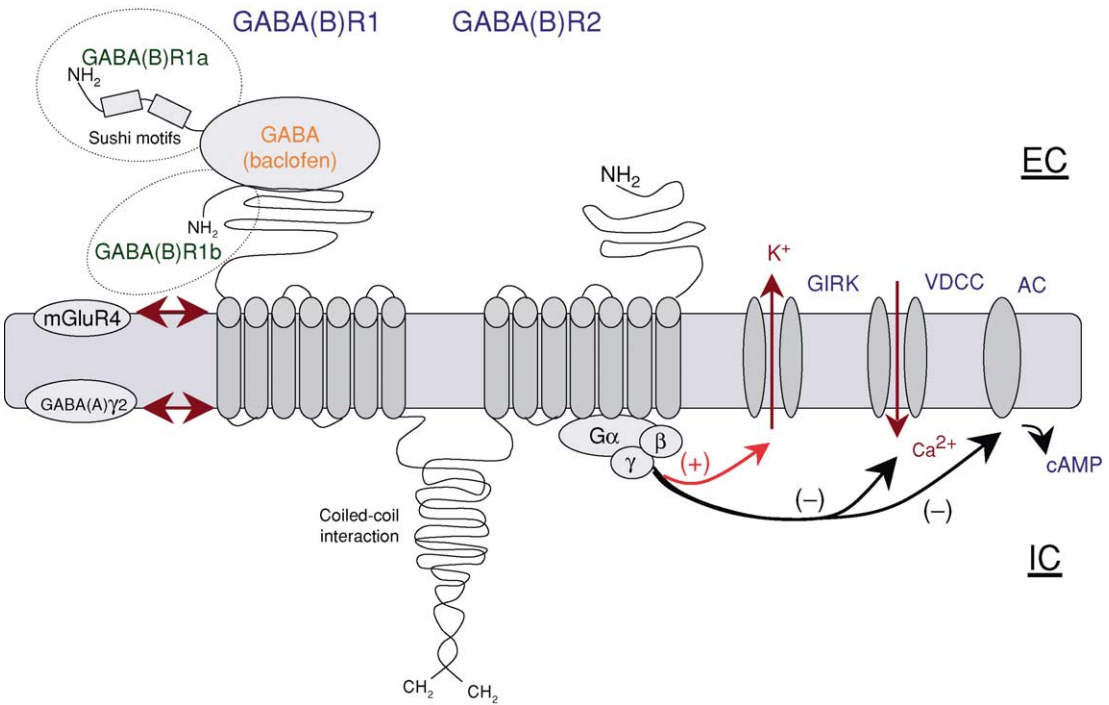

FIG. 1. The $GABA_B$ receptor dimer is composed of $GABA_BR1$ and $GABA_BR2$ subunits. $GABA_BR1$ contains the ligand binding site, while the G-protein complex associates to $GABA_BR2$. Through G-protein-mediated coupling, the activated receptor can regulate inward rectifying K-channels (GIRK), voltage dependent Ca-channels (VDDC), and adenylate cyclase (AC) enzymes. The N-terminal (extracellular) domain of $GABA_BR1a$ contain, Sushi-sequences, while $GABA_BR1b$ does not. Several $GABA_BR1$ subunits can associate with different receptors, among them are $GABA_A\gamma2$ and mGluR4.

$GABA_B$ signaling. The knockout phenotypes provided further evidence for the importance of $GABA_B$ mediated functions in psychiatric disorders.

Box 5

There is some evidence that association of $GABA_{B2}$ with the $GABA_{B1}$ subunit is not absolutely necessary for $GABA_B$ signaling (Gassmann *et al.*, 2004). Also, the broader expression of the $GABA_{B1}$ subunit in comparison to $GABA_{B2}$ may suggest that $GABA_{B1}$ could be functional in the absence of $GABA_{B2}$ (Clark *et al.*, 2000). Besides composing homomer dimers, $GABA_{B1}$ subunits can co-assemble with multiple receptor proteins, among them with $GABA_A\gamma 2$ and mGluR4. For the date, however, no conclusive evidence on the function of chimera receptors has been obtained (Sullivan *et al.*, 2000).

2. *The Expression of $GABA_B$ Receptor During Development*

Despite the functional requirement for both $GABA_{B1}$ and $GABA_{B2}$, the expression of the two subunits seems to be controlled by independent mechanisms, during embryonic development (Martin *et al.*, 2004). Some more important roles for $GABA_{B1}$ over $GABA_{B2}$ are indicated by the earlier and higher expression of $GABA_{B1}$ in the course of development (Kim *et al.*, 2003) (Box 6).

Box 6

$GABA_{B1}$ receptor mRNA but not $GABA_{B2}$ was detected at E11 in the rat hippocampal formation and cerebral cortex (Kim *et al.*, 2003). At E14 both transcripts were expressed, although $GABA_{B1}$ was detected more abundantly. In the prenatal period, both subunits were found in the subplate and the cortical plate, while Cajal-Retzius cells and tangentially migrating cells in the lower intermediate zone contained only $GABA_{B1}$ (Lopez-Bendito *et al.*, 2002). At E17, the two subunits displayed characteristic, region-specific distribution (Behar *et al.*, 2001).

In the rat brain $GABA_{B1a}$ is the prevalent isoform at birth, whereas $GABAB_{1b}$ protein is more abundant in adults (Fritschy *et al.*, 1999). $GABA_{B1a}$ and also $GABA_{B1b}$ proteins were demonstrated in the ventricular zone and cortical plate of the developing rat cortex at E17 (Behar *et al.*, 2001).

The expression of both proteins has a peak in early postnatal development and declines to the adult level thereafter. Receptor binding studies in rat brain also revealed a transient peak during early postnatal development (Turgeon and Albin, 1994).

In comparison to $GABA_A$, *functional* $GABA_B$ receptors appear in later phases of embryonic development. In the neocortex of developing rat brain, $GABA_B$ receptor activity was detected at embryonic day E17 both in ventricular zone and cortical plate (Behar *et al.*, 2001). On differentiating hypothalamic neurons a tonic inhibitory effect of $GABA_B$ receptor signaling was shown as early as E15. The activation of $GABA_B$ receptors depressed both the release of GABA and the $GABA_A$ receptor-mediated Ca2+ rises (Obrietan and van den Pol, 1999).

III. GABA as a Developmental Signal

To date, a growing body of evidence indicates that GABA plays an important regulatory role in the development of the nervous system. The transient GABA production in many regions of the developing CNS underlines the functional importance of GABA in the embryonic and early postnatal neural differentiation (Verney, 2003). Developing neural cells release GABA into their microenvironment by mechanisms still not properly understood (Demarque *et al.*, 2002). While in the mature CNS GABA acts mainly as a synaptic neurotransmitter and elicits phasic responses, in the developing nervous tissue GABA acts as an autocrine/paracrine signal molecule and its main roles are executed through tonic signaling. In young differentiating neurons, most important effects of GABA are mediated through $GABA_A$ receptors and result in membrane depolarization, and in an increased $[Ca^{2+}]_I$, which in turn can stimulate multiple cellular processes (Ohbayashi *et al.*, 1998). $GABA_B$ receptor-mediated effects, which can cause hyperpolarization and reduce $[Ca^{2+}]_I$, appear in later phases of tissue genesis, and seem to balance GABA(A) signaling. The GABA-evoked phasic hyperpolarizations through $GABA_A$ receptors are delayed until the late phases of the maturation of neuronal circuits.

The effects of GABA had been demonstrated in the entire period of neural tissue genesis, from proliferation, through migration and differentiation of neuronal precursors, up to the synapse formation and circuit refinement by maturing neurons.

A. Effects of GABA on the Proliferation of Neural Progenitors

In vertebrate central nervous system, the majority of neurons and all macroglia cells (astrocytes and oligodendrocytes) derive from specialized "germinative" zones (Box 7), which consist of proliferating neuroectodermal progenitor cells (Bayer and Altman, 1987).

Box 7

The early-generated neurons, including some transient "organizer" cells as the Cajal-Retzius cells in the cerebral cortex (Marin-Padilla, 1998), and the projection-type (Golgi-I) large neurons as the spinal motoneurons, or the V-VI layer cortical pyramidal cells, derive from the primary germinative zones (ventricular zone, VZ). With aging, the rate of cell production in the primary germinative zones decreases and the zone transforms to a transport epithelium, the ependymal layer. In most regions, a new layer of proliferating cells forms at the internal face of the transport epithelium, the *subventricular zone* (SVZ) or *secondary germinative zone*. In comparison to the primary germinative layer, SVZ generates less and smaller-size projection-type neurons. The vast majority of SVZ-derived neuronal precursors develop into interneurons. By the perinatal age, SVZ progenitors produce less neuronal and more macroglial precursors.

The *primary germinative zone* is a layer of proliferating neuroepithelial cells, and compose the entire neural tube in early phases of neurogenesis. In the primitive neural tube, neuroepithelial cells span through the total thickness of the tube wall. These radial neuroepithelial cells or embryonic neural stem cells (Gotz *et al.*, 2002) can divide symmetrically and generate identical progenies, and as a result, expand the germinative zone. Alternatively, they can go through asymmetric mitoses, which result in equal distribution of genomic DNA, but unequal distribution of cytoplasmic regulatory components. As a consequence they give rise to two different daughter cells: one, which maintains a mother-like proliferating cell phenotype, while the other is primed for a different cell fate. The later ceases contact with the ventricular wall, situates out from the germinative zone, and stops dividing. These "postmitotic" cells are the earliest neuronal precursors (Jacobson, 1978). With the advancement of embryonic development, the germinative zone gets restricted to a layer lining the brain ventricles and the central canal of the spinal cord.

At the ventricle wall of the future striatum, secondary proliferative zones appear early (E10.5 in rat) and form the primordia of the medial and lateral ganglionic eminences (MGE and LGE, respectively) (Garcia-Verdugo *et al.*, 1998). Precursors of the cortical GABAergic interneurons are generated in the ganglionic eminences (Anderson *et al.*, 1999) and migrate from here to their cortical destination (Kriegstein and Noctor, 2004). The cell production capacity at these sites persists through adulthood. A layer of proliferating cells also persists beneath the granular layer of the dentate gyrus, (hippocampal subgranular layer) and produces granule cell-precursors for the hippocampus throughout the whole life-span (Kaplan and Hinds, 1977). At large areas along the ventricle walls, however, the spontaneous cell-proliferation capacity is exhausted soon after birth. In these areas, severe loss of neural tissue cells can induce some renewal of cell productive capability.

Along the ventricle wall, the mitotic activity and cell-release occur in well-recognizable, ordered, spatial-temporal patterns (Sidman and Rakic, 1973). There are intermitting waves in cell production, and the cell-generation waves are differently timed in adjacent areas (Nornes and Das, 1974). The patterns of the cell production argue for potent regulatory mechanisms, but their nature is not understood.

Cell proliferation can be initiated and is regulated by various growth factors. Epidermal growth factor (EGF) and basic fibroblast growth factor (FGF2) are strong mitogens for neural progenitor cells in different stages of tissue development (Martens *et al.*, 2000). The progression through the cell cycle needs regulated oscillation of $[Ca^{2+}]_I$, which can be evoked by membrane depolarization. The proliferation stimulatory effect of depolarization had been demonstrated in the developing CNS (Cone, 1980).

GABA is a factor, which can elicit membrane depolarization, can evoke increase in the intracellular free Ca-level, can stimulate the release of growth factors and can modulate their effects (Marty *et al.*, 1996). Before the formation of the blood-brain barrier and full functioning of the GABA uptake system, a relatively high GABA-level can be postulated in the environment of proliferating cells. In addition, GABA-containing cells were detected directly above the germinative zone (Cobas *et al.*, 1991), which might release enough GABA to stimulate adjacent cells.

Functional $GABA_A$ receptors had been demonstrated on cells in the germinative zones at various stages of neural development (Ma *et al.*, 1998). LoTurco and co-workers provided the first evidence that the presence of GABA can directly influence the DNA synthesis in proliferating ventricular zone cells. (LoTurco *et al.*, 1995). Activation of $GABA_A$ receptors in the ventricular zone of rat embryos older than E15 significantly reduced the DNA synthesis and resulted in a reduced number of proliferating cells (LoTurco *et al.*, 1995). Comparative studies on cells in different germinative layers of E13-E14 rat forebrain, however, indicated that GABA decreased the cell production in the subventricular zone, but could stimulate the proliferation in the ventricular zone (Haydar *et al.*, 2000). In respect of proliferation stimulatory or inhibitory actions of GABA, some non-congruent results were obtained on postnatal progenitors (Nguyen *et al.*, 2003). The available data (LoTurco *et al.*, 1995) suggest that the proliferation modulating effects of GABA highly depend on the differentiation stage of the targeted cells.

Despite the experimental evidence on the proliferation modulating effects of GABA, GAD 65/GAD 67 double knocked out mouse embryos with a GABA--level less than 0.1% of the normal, did not show severe loss or gain of neurons. While such animals die soon after birth, they failed to display anatomical brain malformations at birth (Ji *et al.*, 1999). The observation shows that even if GABA can modulate the proliferation of neural progenitors, this capability is shared with

a number of other factors and can be replaced in the course of *in vivo* neurogenesis.

B. Effects of GABA on Migrating Neuronal Precursors

Postmitotic neuronal precursors reach their destination by migrating through shorter or longer distances. The migrating cells move along apparently strictly defined routes (Box 8).

Box 8

Postmitotic precursors born in the primary germinative layer leave the zone either by translocation toward the future pial surface through their apical process or by migrating along neighboring radial cells (Kriegstein and Noctor, 2004). Upon leaving the germinative zone, the early-born cells loose mitotic capability and develop into neurons. Cortical interneuron-precursors derived from the subventricular zones may take more complicated routes. Most of them migrate along some tangential guidance points (more or less parallel with the ventricular wall) before they change direction and move radially to their cortical destination.

Path finding is primarily executed by interactions between cell adhesion molecules on the surface of the migrating cell and attachment-points provided by surfaces of adjacent cells and by components of the extracellular matrix. For stable attachment, spreading, or migration, an initial chemical contact has to be established, which in turn can initiate complex cell responses involving recruitment of further adhesion molecules, cytoskeletal activation, incorporation and retraction of membrane-units, and redistribution of several protoplasmic components.

The cell will move toward fields, which provide stronger attachment and produce signals which enhance metabolism and $[Ca^{2+}]_I$. As subpopulations of cells carry different adhesion molecules and deposit different extracellular matrix components, delicate spatial-temporal maps of potential migratory routes are outlined.

The level of free Ca^{2+} is a sensitive intracellular regulator of the formation of the leading lamellipodia and generation of contractile forces needed for active cell displacement. Depolarizing agents, among them GABA, can cause Ca-influx through voltage-dependent Ca-channels, and as a consequence, can modulate the speed and direction of migration. Both too low and too high local $[Ca^{2+}]_I$, however, can prevent locomotion.

In the developing CNS, several GABA sources have been identified, which can provide local GABA and also GABA-gradients for modulation of migration. GABA-containing cells above the germinative zone might produce enough

GABA to influence the migration of postmitotic cells from the proliferative zone. In the marginal zone of the developing cerebral cortex, Cajal-Retzius cells produce GABA, beside the major migration-regulating compound, reelin (D'Arcangelo *et al.*, 1995). In the developing cortical plate, immigrating interneuron-precursors release GABA, which is thought to influence the locomotion of later arriving precursors. Ensheathing glial cells and also the migrating neuronal precursors in the rostral migratory route release GABA (Bolteus and Bordey, 2004). The available data indicate that GABA is an important constituent of the environment of migrating cells.

Migrating neuronal precursors express $GABA_A$ receptors and can respond to GABA by depolarization (Barker *et al.*, 1998). *In vitro*, femtomolar concentrations of GABA were shown to evoke directed displacement, while micromolar quantities caused enhanced but random motility in different developing neural cells (Behar *et al.*, 1996).

In contrast, studies on neurosecretory neurons migrating from the olfactory placode to the basal forebrain indicated that GABA could restrict the cell motility (Fueshko *et al.*, 1998). Activation of $GABA_A$ receptors inhibited the migration of GnRH cells (Heger *et al.*, 2003). Blocking $GABA_A$ receptor signaling, however, disrupted the migratory route and resulted in an ectopic localization of GnRH neurons in the brain (Bless *et al.*, 2000).

The migration inhibiting effect of GABA was also demonstrated on postnatal forebrain neural progenitors (Bolteus and Bordey, 2004). Application of 10 μM GABA reduced, while bicuculline increased the speed of cell migration in the anterior SVZ and in the rostral migratory stream. In higher (100 μM), desensitizing concentration, GABA increased the motility by auto-blocking the GABA-elicited inward anion-flow. The results clearly demonstrated that $GABA_A$ signaling could decrease or increase the speed of migration depending on the local GABA-concentration (Bolteus and Bordey, 2004). Moreover, GABA-induced depolarization can elicit further GABA-release, and the outcome of this GABA-loop may determine the inhibition or relief from inhibition of motility. In this system, IP_3-sensitive Ca-stores were suggested as a source of free Ca^{2+}, rather then depolarization-evoked influx of Ca^{2+} (Bolteus and Bordey, 2004).

GABA can modulate the migration of neuronal precursors via $GABA_B$ receptor-mediated effects, as well. Behar and co-workers (Behar *et al.*, 2000) demonstrated that while migration from the ventricular zone to the intermediate zone of E18 rat forebrain was modulated via $GABA_A$ receptors signaling, the migration toward the cortical plate was regulated by $GABA_B$-mediated processes. Involvement of $GABA_B$ signaling in the migration toward the cortical plate was also demonstrated by the accumulation of tangentially migrating cells in the ventricular zone of E15 rat embryos in the presence of $GABA_B$ antagonists

(Lopez-Bendito *et al.*, 2003). As $GABA_B$ signaling decreases depolarization and $[Ca^{2+}]_I$, one can assume that it can prevent the over-activation and consequent disruption of the cytoskeletal locomotory machine.

In spite of much *in vitro* and *in vivo* evidence on the migration-modulating effects of GABA, the cytoarchitecture of the GABA-deficient neonatal brain failed to show severe malformations (Ji *et al.*, 1999). The observation shows that the vital processes of correct cell migration are governed by redundant regulatory mechanisms, which can compensate for the shortage of a single factor.

C. Effects of GABA on Growth Cone Motility and Neuronal Process Elongation

During migration, neuronal precursors have transient leading lamellipodia and pulled tails, as do any other migrating tissue cells. The *neuron-specific polarization*, namely the formation of a permanent process (future axon) on a defined part of the cell (neuritogenesis), starts usually after the settlement of the cell (Goffinet, 1984). The mechanisms resulting in the selection of one of the minor processes as the rudiment of the future axon (Dotti *et al.*, 1988) and in *the initial outgrowth* of the axon are not fully understood. Growth factors, mainly the members of the neurotrophin family, NGF (Greene *et al.*, 1984) and BDNF (Avwenagha *et al.*, 2003), were shown to promote the initial outgrowth of neurites. GABA administration was demonstrated to evoke growth factor release and also to provoke process outgrowth from cells of superior cervical ganglia (Wolff *et al.*, 1978).

The *elongation of the neurite* is persecuted by adhesion-dependent advancing of its leading edge, the growth cone, and by the orchestrated incorporation of axonal constituents right behind the growth cone. The process elongation will sensibly respond to a variety of intra- and extracellular signals and will be the subject of multiple regulations (Xiang *et al.*, 2002) (Box 9). Changes in the local concentrations of cAMP, cGMP, and Ca^{2+} were all shown to play determining roles in the growth cone responses to environmental signals indicating the involvement of multiple surface receptors in both the advancement and collapse of a growth cone (Cooper, 2002).

The advancement, halt, turn, or collapse of a growth cone well correlate with the spatial-temporal pattern of its internal free calcium concentration ($[Ca^{2+}]_I$) (Henley and Poo, 2004). The repulsive attachment-signals will either evoke an over-range increase in $[Ca^{2+}]_I$ or prevent the elevation of Ca-level. Besides the ECM and cell surface components, a large variety of secreted compounds, among them GABA and glutamate, can regulate growth cone advancement by interfering with the $[Ca^{2+}]_I$ (Obrietan and van den Pol, 1996).

Box 9

Growth cone advancement is regulated by evolutionary conserved receptor-ligand pairs (see Table I), which regulate the Ca-level at the site of attachment. These attachment-receptor systems interfere with each other in deciding the actual growth cone response.

Growth cone advancing requires the incorporation of membrane material, which is delivered by the fusion of exocytotic vesicles with the plasma membrane (Bray, 1973). The fusion needs defined (~0.5 μM) local $[Ca^{2+}]_I$ (Burgoyne and Morgan, 1995). The driving force generation by actin-myosin complexes also needs Ca^{2+}, and proceeds only at those sites of the cone which possess Ca^{2+} in the right concentration. These mechanisms result in turning the axon toward signal sources, which can provoke an appropriate local rise in $[Ca^{2+}]_I$ (Gomez *et al.*, 2001).

The microtubule system expanding into the central (but not to peripheral) part of the growth cone provides the "conveyor" for the transportation of vesicles, actin monomers, and other constituents to the growth cone (Gordon-Weeks, 2004). The maintenance and transport functions of the microtubule bundles need Ca^{2+}, but the assembly is getting corrupted at high (>1 μM) $[Ca^{2+}]_I$. High $[Ca^{2+}]_I$ in the central part results in rapid collapse of the growth cone.

TABLE I

Growth cone receptor	Ligand	Attachment signal	Reference
DCC/Unc5	Netrins	repulsive/attractive	Wadsworth *et al.*, 1996
Robo	Slit	repulsive	Wong *et al.*, 2002
Neuropilins	Semaphorins	repulsive/attractive	Chen *et al.*, 1998
Eph (Trk receptors)	Ephrins	repulsive/attractive	Himanen and Nikolov, 2003
Nogo	MAG, OMgp	repulsive	McGee and Strittmatter, 2003

The presence of functional $GABA_A$ receptors has been demonstrated on growth cones of many types of neurons and their activation results in a local increase in $[Ca^{2+}]_I$ (Fukura *et al.*, 1996). Growth cones are known to release GABA, and can actively regulate the GABA-concentration in their vicinity (Lockerbie *et al.*, 1985). The available data suggest that GABA acts as an autocrine factor for the majority of the growing neurites. The auto-release serves to keep the basic Ca-concentration at a slightly elevated level, which in turn makes the cone more sensitive for other signals.

Besides $GABA_A$ receptors, $GABA_B$ receptors are also present in the growth cone membrane of various types of neurons (Xiang *et al.*, 2002). Selective stimulation of $GABA_B$ receptors by baclofen inhibits growth cone advancement (Priest and Puche, 2004) by decreasing the internal $[Ca^{2+}]_I$ (Behar *et al.*, 2001).

$GABA_B$ receptor-mediated signaling may prevent the potential over-excitation caused by autocrine or environment-derived GABA and may help to maintain an optimal sensitivity level of the growth cone.

Through $GABA_A$ and $GABA_B$ signaling, GABA can influence the motility—advancement, turning, or collapse—of growth cones, and can regulate the elongation and path-finding of neurites. Despite the convincing experimental data, however, the *in vivo* consequences of the lack of GABA are less clear. Data obtained on animals lacking one (GAD 67) (Asada *et al.*, 1997) or both (GAD 67 and GAD 65) (Ji *et al.*, 1999) GABA-synthesizing enzyme, and born with a highly reduced brain GABA-level (7% and 0.02% of the normal, respectively), failed to demonstrate major alterations in the fiber maps of the CNS at birth.

D. The Role of GABA in Synaptogenesis and Synapse Stabilization

Elongating neuronal processes can travel for a long distance and pass or make only transient contacts with multiple potential synaptic partners. In the main period of process-elongation, developing axons produce excess numbers of collateral branches and grow into many areas from which they will diminish in later phases of functional network formation (Stanfield, 1984). While synapses are formed (and diminish) during the whole lifetime, the main periods of synaptogenesis in defined regions are confined to relatively narrow time-windows (critical periods) when most future innervating axons arrive at the potential target-area. Recordings made on various regions of the developing CNS (O'Donovan, 1999) demonstrated ongoing, periodic electrical activities and "spontaneous" Ca-spikes (Spitzer *et al.*, 2004) during the formation of synaptic circuits (Box 10). In the developing neural tissue, large depolarizing waves (also called as giant depolarizing potential, GDP) (Ben-Ari *et al.*, 1989) are propagated along the membranes of the differentiating cells and their processes, in spatially and temporally organized ways (Katz and Shatz, 1996), providing time- and space windows for synaptogenesis.

The travelling waves, by providing transient Ca-level elevations in *a* growth cone or in *a* potential future postsynaptic patch, are not enough for the initiation of synapse formation, but make the compartments sensitive for additional extracellular signals. The majority of the exogeneous signals—comprising growth factors, matrix molecules, and Ca-elevating neurotransmitters (Box 11)—are produced and released by the potential synaptic partners, upon activation. The inherent Ca-waves and the pattern of exogeneous signals together will decide on synapsing or passing by the actual partners.

Box 10

The waves presumably are generated by an initial fast Ca-spike, which evokes Ca-induced Ca-release, locally. In the developing spinal cord, periodic waves with amplitudes of 10–40 mV and with durations in the range of several 10 seconds can result in local Ca-oscillations reaching μM levels of $[Ca^{2+}]_I$ for short but repeated periods (Gu and Spitzer, 1997). Elevated free Ca^{2+} concentration is necessary for the formation of synaptic compartments (see below).

Coinciding activity of the pre- and postsynaptic partners is inevitable for the formation and also for the maintenance of a synapse and the establishment of the mature "process-map." Depolarization travelling along the developing neuronal processes is thought to play a pivotal role in the establishment of the topographic projections between innervating and target fields. The depolarization is thought to derive from pacemaker neurons, which are electrically coupled to a defined number of their neuron-mates. In this way, a number of neighboring cells get activated in a rhythm corresponding to the parameters of the spontaneous depolarization of the pacemaker, while others will adopt a different rhythm. Axons originating from a coupled group of neurons will "fire" and release factors at their endings with an almost identical timing. At the termini of bundles of axons, the concentration of synchronously released factors can rise to a level sufficient to transactivate an area where the potential postsynaptic partners are located. Activated "targets" will produce signal molecules and will be ready to respond to an initial contact by postsynaptic specification. In the same fasciculated axon bundle, there are neurites derived from neurons of a different coupling group. Their endings will be silent in those periods when the other coupling group activates the target-region. In the lack of synchronous activity, they will establish synapses at lower probability and get excluded from the given target field. This schedule of synchronous activity driven innervation gives an explanation of the formation of topographic projections, and is referred to as an example of the principle of "fire together, wire together."

Synaptogenesis is an interactive process between the future pre- and postsynaptic element (Jessell and Kandel, 1993) (see Fig. 2). Synapse formation starts with an initial membrane contact between the potential partners, and ends up in the formation of complex, functional molecular assemblies, including the active release-area in the presynaptic side, the signal-processing machinery (Kennedy, 2000) at the postsynaptic site, and the structured extracellular matrix in the synaptic cleft (Patthy and Nikolics, 1994). The initial membrane contact is a mutual signal for both partners, which, depending on the actual physiological status of the cell compartment, can or cannot initiate the assembling of synapse-building molecules. After the initial contact, the potential synaptic partners cease locomotory membrane movement for a period of 10 minutes (Pfenninger

Box 11

Specific cell adhesion molecules were shown to govern initial coupling and forthcoming molecular events on both sites (Biederer *et al.*, 2002). The assembly of pre- and postsynaptic specializations is beyond the scope of this chapter. We refer to the excellent reviews of Ziv and Garner (2004), respectively. Pre-assembled, multimolecular units are delivered to both the pre- (Krueger *et al.*, 2003) and postsynaptic active zones by intracellular transport. Many synaptic proteins, however, are synthesized locally, at the nerve-endings (Schratt *et al.*, 2004). Local protein synthesis in both developing and mature postsynapses has been widely demonstrated. Indirect evidence for protein translation in the presynaptic terminals exists, but direct evidence was obtained only on invertebrate neurons (Martin, 2004).

The rate of the local protein production (Kalinovsky and Scheiffele, 2004) and the speed of delivery and incorporation of assembled units are regulated by growth factors and bioelectric activity (Sutton *et al.*, 2004). Increase in the $[Ca^{2+}]_I$ of the developing endings results in the exocytotic release of various factors, among them BDNF, which in turn can directly elevate the local protein synthesis (Schratt *et al.*, 2004) by signaling through cell surface tyrosine receptor kinases.

In the course of synapse-formation, the extracellular space between the partner-membranes widens gradually (Pfenninger and Maylie-Pfenninger, 1979) from an initially close apposition to a wide gap of about 200 nm. The synaptic cleft contains specific extracellular matrix components, and many of them play roles in the clustering of pre- and postsynaptic membrane components (Mi *et al.*, 2002).

and Maylie-Pfenninger, 1979). In this time-window, either the deposition of synapse-specific material starts or the locomotion activity of the membranes gets reactivated and the partners separate from each other.

Synapse-formation can take place only if both partners are activated and display synchronously elevated $[Ca^{2+}]_I$. The increase in the local $[Ca^{2+}]_I$ is mainly achieved by local Ca-influx through depolarization-activated Ca-channels. Besides the propagated bioelectric waves, developing neuronal membranes can be further depolarized by binding neurotransmitters, mainly GABA and glutamate through $GABA_A$ and NMDA receptors, respectively (Ben-Ari, 2002). Elements for functional synapses seem to be stored and kept ready for rapid incorporation in yet silent synapses of developing neurons (Krueger *et al.*, 2003). In developing hippocampal slices, rhythmic depolarization resulted in the formation of functioning synapses in 30 minutes (Gubellini *et al.*, 2001).

GABA seems to be the main, intrinsic depolarizing agent in the critical periods of neuronal circuit formation in many areas of the developing CNS. Using $GABA_A$ receptor antagonists, the role of GABA in generation of large

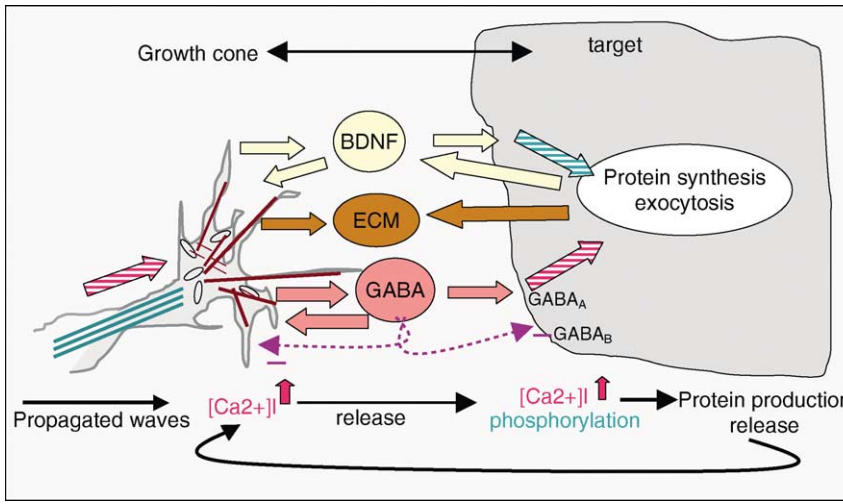

FIG. 2. Depolarization increases the intracellular Ca-level at the growth cone and elicits the release of growth factors (in most cases BDNF), extracellular matrix molecules (ECM), and GABA. BDNF through Trk receptors will stimulate local protein synthesis in both partners resulting in the production of further factors to be released. GABA also will depolarize both partners and trigger the release of various factors, among them BDNF, ECM molecules, and neurotransmitter substances, mainly GABA. The over-excitation by increasing concentrations of GABA can be prevented by the activation of $GABA_B$ receptors, which will reduce or even stop depolarization.

depolarizing waves was demonstrated in the areas of the developing hippocampus (Ben-Ari *et al.*, 1989), retina (Fischer *et al.*, 1998), and spinal cord (O'Donovan *et al.*, 1998). GABA-regulated Ca-spikes were recorded also in the developing cerebral cortex (Katz and Shatz, 1996) involving the early marginal zone (layer 1) containing Cajal-Retzius cells (Schwartz *et al.*, 1998).

GABA can exert these effects through both extrasynaptic and synaptic $GABA_A$ receptors. In a developing neural circuit containing neurons and contacts at different stages of differentiation, the paracrine *versus* neurocrine modes of actions are hardly distinguishable. Depolarizing GABA signaling through either synaptic or extrasynaptic $GABA_A$ receptors can elicit further neurotransmitter release, and creates positive feedback loops which will favor the maintenance of the activation (see also Fig. 2).

In periods and sites of massive synapse formation, GABA can act also through $GABA_B$ receptors. Signaling through $GABA_B$ receptors will reduce depolarization by activating $GABA_B$ receptor-coupled K-channels. This counter-action to $GABA_A$ signaling, together with the desensitization of $GABA_A$ receptors by extreme GABA-concentrations, can prevent a GABA-induced over-activation and provide a mechanism for rhythm generation.

Regional analyses demonstrated that GABAergic signal-machinery develops earlier then the other neurotransmitter signaling apparatuses in a local network (Schwartz *et al.*, 1998). GABA-synapses were shown to develop prior to glutamatergic ones in the developing hippocampus and also in the cerebral cortex (Ben-Ari *et al.*, 2004). The formation of more targeted, spatially restricted neurocrine communication-apparatuses will limit the activated cells and helps to shape the local network. The targeted and coinciding activation will speed up the synapse formation between activated cells and will help their process arborization, as well. By incorporating cell-specific, pre-formed synaptic elements into synaptic specializations, GABA-induced depolarizations can elicit the formation of glutamatergic (Ben-Ari, 2002) and presumably other neurotransmitter-operated synapses. In the developing spinal cord, the frequency and the magnitude of the depolarizing waves were suggested to regulate the transmitter-phenotype-choice of the activated cells (Spitzer *et al.*, 2004).

Ongoing activity elicits fundamental changes in maturing neurons. In parallel with synapse-formation, the sets of ion-transporters change. With development of Cl^--extruding mechanisms, $GABA_A$ receptor signaling will evoke hyperpolarization instead of depolarization (Rivera *et al.*, 1999). By the period of the bulk formation of excitatory synapses, GABAergic signaling turns to inhibitory and provides a potent protection against toxic over-excitation.

GABA-signaling, with its inherent, auto-regulated biphasic potential to hypo- and hyperpolarize the target cells, and also with the mild (non-toxic) shifts it can cause in the ion-homeostasis, is a potent candidate for the role of a developmental activity-generator and neuronal circuit-organizer.

Unfortunately, animals lacking functional GABA-synthesizing enzymes, GAD65 and GAD67 (Ji *et al.*, 1999), die soon after birth, and cannot provide tools to study the relatively late events of functional network formation. Further studies should decide whether GABA-effects are dispensable in the processes of precise wiring and network formation, as well as in the determination of the neurotransmitter phenotype of differentiating neurons.

IV. Concluding Remarks

A large body of experimental evidence demonstrates that GABA-signaling possess an inherent capability for potent regulation of almost all steps of neuronal differentiation and neural tissue formation. Several data also suggest that a transient GABA-production is an inherent feature of many differentiating neuronal populations, regardless of the future neurotransmitter phenotype. The apparently normal prenatal brain development in GABA-deficient animals, however, indicates that GABA can be replaced by other regulatory factors in modulating neural cell

production, neuronal precursor migration, process outgrowth, path finding, and neurite elongation. The most intriguing developmental role that has been attributed to GABA is the generation and maintenance of activity waves in the period of functional network formation. For the time being, there are no tools to approach this question at the level of the whole organism experimentally. Accumulating pathophysiological data on inherent developmental neurological, psychiatric, and mental disorders, however, may help to reach convincing conclusions.

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GABAERGIC SIGNALING IN THE DEVELOPING CEREBELLUM

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In the adult central nervous system (CNS), γ-amino butyric acid (GABA) is the predominant inhibitory neurotransmitter, and it regulates glutamatergic activity. Recent studies have revealed that GABA serves as an excitatory transmitter in the immature CNS, and acts as a trophic factor for brain development. Furthermore, normal formation of GABAergic synapses is crucial for the expression of higher brain functions such as memory, learning, and motor coordination, and various psychiatric diseases such as anxiety disorders, epilepsy, schizophrenia, and autism are partly caused by the dysfunction of GABA in the developing and mature brain. These results indicate that the GABAergic roles change developmentally with special references to alterations in GABAergic transmission and signaling, and that GABA plays various roles in the expression of almost all brain functions. We morphologically investigated the developmental changes in the GABAergic transmission system and the key factors for the

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formation of GABAergic synapses and networks. Here, we focus on the cerebellar cortex, which provides an ideal system for the investigation of brain development, and review four points: (1) The GABAergic system in the adult cerebellum, (2) GABAergic signaling before synaptogenesis—the mechanisms by which GABA exerts its effect on developing neurons, (3) Formation of GABAergic synapses—mechanisms underlying formation of functional GABAergic synapses, and (4) Functions of GABAergic synapses.

I. Introduction

In the mammalian central nervous system (CNS), GABA is the predominant neurotransmitter, and it plays various roles in the expression of brain functions by activation of ionotropic and metabotropic GABA receptors. In the adult CNS, GABA mediates inhibitory synaptic transmission, regulates glutamatergic activity, and prevents hyperexcitation (Kardos, 1999; Macdonald and Olsen, 1994; Olsen and Avoli, 1997). In the developing CNS, GABA acts as trophic factor, and induces brain morphogenesis, including changes in cell proliferation, cell migration, axonal growth, synapse formation, steroid-mediated sexual differentiation, and cell death (Barker *et al.*, 1998; Belhage *et al.*, 1998; Ben-Ari, 2002; Kardos, 1999; McCarthy *et al.*, 2002; Owens and Kriegstein, 2002; Varju *et al.*, 2001). Furthermore, during the maturation period, GABAergic transmission controls experience-dependent plasticity in the visual cortex, induces long-term potentiation, which is the electrophysiological basis of memory and learning (Ben-Ari *et al.*, 1997; Fagiolini and Hensch, 2000; Freund and Gulyas, 1997; Hensch *et al.*, 1998; Kardos, 1999; McBain and Maccaferri, 1997; Paulsen and Moser, 1998; Wolff *et al.*, 1993), modulates anxiety (Nutt *et al.*, 1990; Pratt, 1992), and generates circadian rhythms (Nutt *et al.*, 1990; Pratt, 1992; Turek and Van Reeth, 1988; Wagner *et al.*, 1997). Various psychiatric diseases such as epilepsy (Avoli, 2000; Baulac *et al.*, 2001; Snead *et al.*, 1999; Wallace *et al.*, 2001), anxiety disorders (Freeman *et al.*, 2002; Millan, 2003; Nutt, 2001), schizophrenia (Berry *et al.*, 2003; Blum and Mann, 2002; Byne *et al.*, 1999; Caruncho *et al.*, 2004; Costa *et al.*, 2004; Lewis *et al.*, 2004; Wassef *et al.*, 2003), and autism (Blatt *et al.*, 2001; Cook *et al.*, 1997; DeLorey *et al.*, 1998; Dhossche *et al.*, 2002; Dhossche, 2004; Fatemi *et al.*, 2002; Lauritsen *et al.*, 1999; Rolf *et al.*, 1993) are partially caused by GABA dysfunction in the developing and mature brain.

The developmental shift in the action of GABA is based on an alteration in GABAergic transmission and signaling. During development, the GABA transmitter system changes from non-synaptic to synaptic mechanisms (Attwell *et al.*, 1993; Fon and Edwards, 2001; Owens and Kriegstein, 2002; Taylor

and Gordon-Weeks, 1991; Varju *et al.*, 2001). The subunit compositions and localization of ionotropic GABA receptors drastically change (Araki *et al.*, 1992; Fritschy *et al.*, 1994; Gambarana *et al.*, 1990; Laurie *et al.*, 1992a; Ma and Barker, 1995; Maric *et al.*, 1997). Environmental changes, such as decreasing intracellular chloride concentration, influence the response of GABA receptors (Ben-Ari, 2002; Cherubini *et al.*, 1991; Ganguly *et al.*, 2001; Owens and Kriegstein, 2002; Perkins and Wong, 1997; Rohrbough and Spitzer, 1996; Serafini *et al.*, 1998). In the first half, we review the GABAergic system in the cerebellum and the developmental changes in GABAergic signaling, and discuss how GABA exerts its effect on immature neurons during development. Establishment of GABAergic synapses is crucial for the expression of normal and higher brain functions. In the second half, we address the key factors for the formation of functional GABAergic synapses, and discuss the mechanisms underlying the formation of GABAergic synapses and networks.

II. GABAergic System in the Cerebellar Cortex

A. GABAergic Neurons and Synapses

The cerebellar cortex consists of molecular, Purkinje cell, and granular layers (Ito, 1984; Llinas and Walton, 1990; Palay and Chan-Palay, 1974). Each layer contains distinct types of neurons. Stellate cells are scattered in the molecular layer, and basket cells are localized in the deep part of the molecular layer. Cell bodies of Purkinje cells are arranged in a single layer between the molecular and granular layers. Numerous granule cells occupy the granular layer and Golgi cells are localized mainly in the upper half of the granular layer. Among the five types of main neurons, four of them, Purkinje, stellate, basket, and Golgi cells, release GABA as a neurotransmitter (see Fig. 1A, B). The GABAergic neurons and the neural circuits in the cerebellar cortex are summarized in Fig. 1B (Ito, 1984; Llinas and Walton, 1990; Palay and Chan-Palay, 1974).

The Purkinje cell is a pivotal neuron in the cerebellar cortex. Each dendrite spreads out in a single vertical parasagittal plane in the molecular layer, and receives excitatory inputs from climbing and parallel fibers at the spines, and inhibitory inputs from stellate cells at the shafts. Cell bodies form GABAergic synapses with the pericellular baskets of basket cell axon collaterals. The Purkinje cell axons traverse the granular layer into the white matter, and innervate the deep cerebellar and vestibular nuclei. In addition, recurrent collateral branches arise from the third or fourth nodes of Ranvier (Cajal, 1911), ramify in the upper granular layer, and give rise to plexuses beneath the Purkinje cell bodies. In the plexus, varicosities of axon collaterals form GABAergic synapses with Purkinje

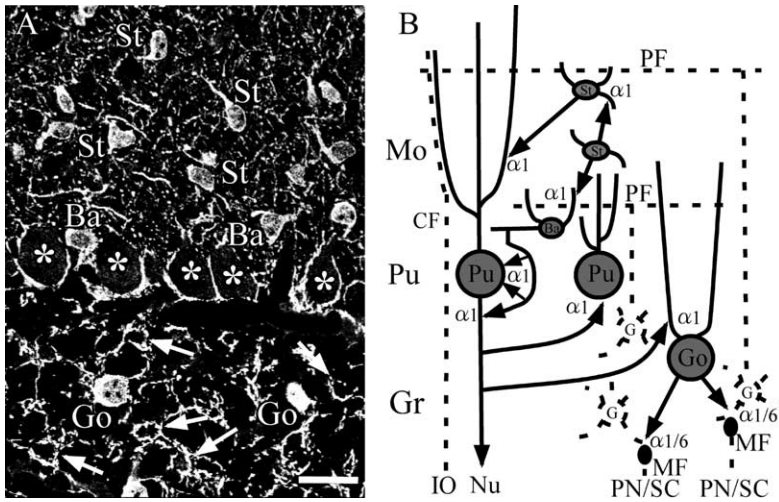

FIG. 1. GABAergic neurons (A) and the GABAergic local circuit in the cerebellar cortex (B). (A) Immunohistochemistry for GABA in the adult mouse cerebellar cortex. Cell bodies and axon terminals of stellate (St) and basket (Ba) cells are labeled in the molecular (Mo) and Purkinje cell (Pu) layers. Golgi cell bodies (Go) and their axon terminals (white arrows) are also stained in the granular layer (Gr). However, cell bodies (asterisks) and dendrites of Purkinje cells are negative. (B) Schematic illustration of the local neural circuit and $GABA_A$ receptor α subunit expression in the adult cerebellar cortex. Abbreviations and symbols. St: stellate cell, Ba: basket cell, asterisk: Purkinje cell body, Go: Golgi cell, white arrow: GABA-positive rings in the synaptic glomerulus, Mo: molecular layer, Pu: Purkinje cell layer, Gr: granular layer, PF: parallel fiber, α1: $GABA_A$ receptor α1 subunit, G: granule cell, α1/6: $GABA_A$ receptor α1 and α6 subunits, MF: mossy fiber, CF: climbing fiber, IO: inferior olivary nucleus, Nu: deep cerebellar nucleus, PN/SC: pontine nucleus and spinal cord, black arrow: GABAergic innervation and synapse, gray circle: GABAergic neuron, dotted circle: excitatory neuron. Bar: 10μm.

and Golgi cells at the cell bodies and dendrites (Palay and Chan-Palay, 1974; Takayama and Inoue, 2004a).

Each stellate and basket cell extends its dendrite only in a parasagittal plane parallel to the fan of the Purkinje cell dendrite within the molecular layer. They receive excitatory inputs from parallel and climbing fibers and inhibitory inputs from stellate cells. The stellate cell axons arborize within the molecular layer, and their varicosities make many GABAergic synaptic contacts with the dendritic shafts of Purkinje cells and other GABAergic neurons, including stellate, basket, and Golgi cells. Basket cell axons run deep in the molecular layer just above the cell bodies of Purkinje cells. Axon collaterals descend along the Purkinje cell dendrites, surround the Purkinje cell bodies, give rise to pericellular baskets, and form periaxonal plexuses, '*pinceau,*' around the initial segment of the Purkinje cell axon (Cajal, 1911). At the pericellular basket, axo-somatic synapses are formed

with Purkinje cell bodies. At the *pinceau*, axo-axonic synapses are formed with the initial segments of Purkinje cells. Both synapses are GABAergic. One basket cell axon innervates about 10 Purkinje cells.

Golgi cells extend their dendrites into the molecular layer. Each Golgi cell dendrite is not confined to a single plane, but opens out into a three-dimensional ungulated field. They receive excitatory input from parallel and climbing fibers in the molecular layer and mossy fibers in the granular layer. Inhibitory inputs come from stellate cells in the molecular layer and Purkinje cell axon collaterals in the granular layer. One to three axons arise from the cell body and main dendrite, divide repeatedly, and give rise to plexuses. At the plexuses, varicosities of Golgi cell axons form GABAergic synapses with granule cell dendrites at the peripheral part of the glomeruli in the granular layer.

The pivotal neurons of the cortex, Purkinje cells, receive excitatory inputs from climbing fibers and granule cell axons, parallel fibers, and send inhibitory output to the deep cerebellar nucleus. GABAergic neurons, stellate, basket, and Golgi cells, negatively regulate above the major stream of cortical circuits at the Purkinje cell dendrites, cell bodies, and granule cell dendrites, respectively.

B. GABA and GABA Receptors

In the CNS, GABA is synthesized from glutamate by two isoforms of glutamic acid decarboxylase (GAD65 and GAD67) (Barker *et al.*, 1998; Martin and Rimvall, 1993; Varju *et al.*, 2001), and is loaded into vesicles by the vesicular GABA transporter (VGAT) (see Fig. 2) (Fon and Edwards, 2001; McIntire *et al.*, 1997; Reimer *et al.*, 1998). In response to the influx of calcium ion via a voltage-dependent calcium channel, GABA is released by the fusion of vesicles with the presynaptic membrane at the nerve terminals, and activates GABA receptors at the postsynaptic membrane. GABAergic signals are terminated by reuptake of neurotransmitter into nerve terminals or uptake into surrounding glia by plasma membrane GABA transporters (GATs) (Cherubini and Conti, 2001).

GABA receptors are classified into three groups on the basis of pharmacology and biochemistry; $GABA_A$, $GABA_B$, and $GABA_C$. Among them, fast synaptic transmission is mediated by ionotropic GABA receptors, $GABA_A$ and $GABA_C$ receptors (Bormann, 2000; Kaupmann *et al.*, 1998; Macdonald and Olsen, 1994; Mehta and Ticku, 1999). The $GABA_A$ receptor is a member of the ligand-gated ion channel receptor family, and is thought to be composed of five heteromeric subunits belonging to seven different subunit families; α1–6, β1–3, γ1–3, δ, ε, π, and θ (Macdonald and Olsen, 1994; Mehta and Ticku, 1999; Nayeem *et al.*, 1994; Olsen and Tobin, 1990; Sieghart, 1995; Sieghart *et al.*, 1999; Tretter *et al.*, 1997). Native $GABA_A$ receptors contain at least one α-, one β-, and one γ-subunit with the δ-, ε-, π-, and θ-subunits to substitute for the γ subunit

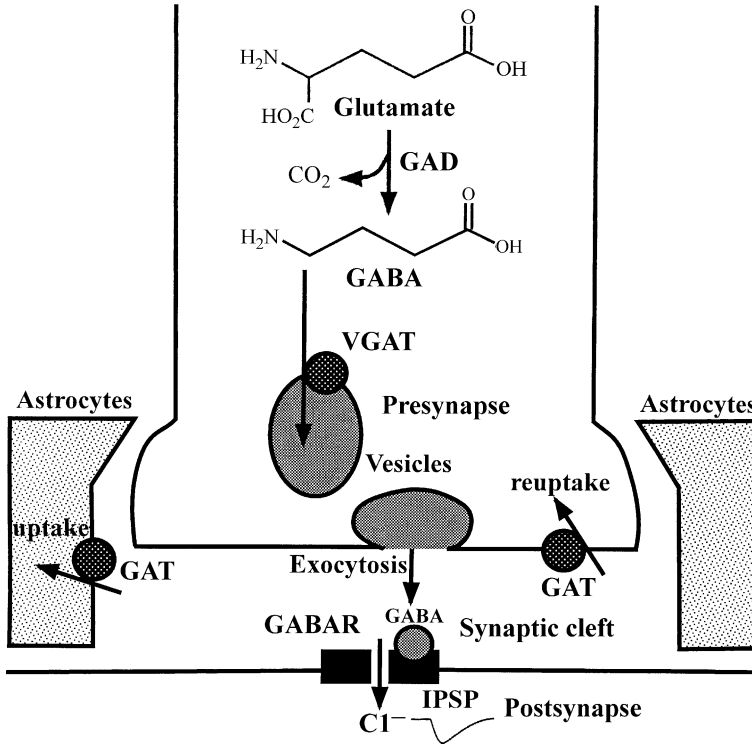

FIG. 2. Schematic illustration of GABAergic transmission in the mature GABAergic synapse. GABA is synthesized from glutamate by glutamic acid decarboxylase (GAD), and is loaded into vesicles by the vesicular GABA transporter (VGAT). GABA is released by the fusion of vesicles with the presynaptic membrane at the nerve terminals, and activates GABA receptors (GABAR) at the postsynaptic membrane. In the adult synapses, activation of $GABA_A$ receptors mediates hyperpolarization of postsynaptic membrane potential (IPSP) by the influx of chloride ion (Cl^-). GABAergic signals are terminated by uptake and reuptake of neurotransmitter into nerve terminals or uptake into surrounding glia by the plasma membrane GABA transporters (GAT). Abbreviations and symbols. GAD: glutamic acid decarboxylase, VGAT: vesicular GABA transporter, GAT: (plasma membrane) GABA transporter, GABAR: GABA receptor, IPSP: inhibitory postsynaptic potential.

(McKernan and Whiting, 1996; Pritchett *et al.*, 1989; Sieghart, 1995; Sieghart *et al.*, 1999). The subunit compositions drastically change during brain development (Araki *et al.*, 1992; Gambarana *et al.*, 1990; Laurie *et al.*, 1992a), and exhibit characteristic pharmacological and electrophysiological properties (Kardos, 1999; Luddens *et al.*, 1990; Macdonald and Olsen, 1994; Olsen and Tobin, 1990; Pritchett *et al.*, 1989; Sieghart, 1995; Vicini, 1999). GABA binding opens the pore of GABA receptors and induces influx or efflux of anions such as chloride ion (Fig. 2) (Kardos, 1999; Macdonald and Olsen, 1994; Olsen and

Tobin, 1990; Sieghart, 1995). The $GABA_C$ receptor is also an ion-channel type receptor, which is composed of only single or multiple ρ subunits. The $GABA_C$ receptor is identified as a bicuculline and baclofen insensitive GABA receptor, and is considered to be a pharmacological variant of $GABA_A$ receptors (Bormann, 2000; Bormann and Feigenspan, 1995; Mehta and Ticku, 1999). The $GABA_B$ receptor, which includes three isoforms, R1a, R1b, and R2 (Kaupmann *et al.*, 1997; Kaupmann *et al.*, 1998), is a metabotropic receptor, activates G proteins, negatively regulates the second messenger system, and responds to slow acting inhibition of channel and receptor functions (Bormann, 1988; Connors *et al.*, 1988; LeVine, 1999; Nicoll, 1988).

C. $GABA_A$ Receptors in the Cerebellar Cortex

All neurons in the cerebellar cortex express the $GABA_A$ receptors. The main composition of the $GABA_A$ receptors is $\alpha1\beta2\gamma2$ (Fritschy and Mohler, 1995; Laurie *et al.*, 1992a; Persohn *et al.*, 1992). In addition, Purkinje cells abundantly express the $\beta3$ subunit ($\alpha1\beta2/3\gamma2$), and granule cells abundantly express the $\alpha6$, $\beta3$, and δ subunits ($\alpha1/6\beta2/3\gamma2/\delta$) (Fig. 1B).

D. Plasma Membrane GABA Transporters

Plasma membrane GABA transporters (GATs) are high-affinity, sodium (Na^+) and chloride (Cl^-)-dependent transporters, and GABA is co-transported with Na^+ and Cl^- (Borden, 1996; Conti *et al.*, 2004; Gadea and Lopez-Colome, 2001; Kanner, 1994). Molecular cloning has isolated four GATs, GAT-1, GAT-2, GAT-3, and BGT-1. (Mouse GAT2, GAT3, and GAT4 are the species homolog of rat BGT-1, GAT-2, and GAT-3, respectively.) They exhibit characteristic distributions in the CNS, including the cerebellum (Durkin *et al.*, 1995; Itouji *et al.*, 1996; Morara *et al.*, 1996; Ribak *et al.*, 1996; Rosina *et al.*, 1999). GAT-1 is mainly localized at the axon terminals containing GABAergic vesicles and is partly detected at the astrocytes (Fig. 3A, B). In contrast, GAT-3 is localized at the processes of astrocytes in the granular layer (Fig. 3C, D). GAT-2 is only localized at the leptomeningeal, ependymal cells, and choroids plexus (Conti *et al.*, 1999).

In the adult CNS, GATs clean GABA from the synaptic cleft into the presynapse or surrounding glia (Fig. 2). In the immature brain or under abnormal condition such as ischemia and seizure, on the other hand, GATs work in reverse, releasing neurotransmitters (Attwell *et al.*, 1993; Levi and Raiteri, 1993), in a calcium independent mechanism. It is thought that this phenomenon induces the development and protects against the effects of seizures (Phillis *et al.*, 1994).

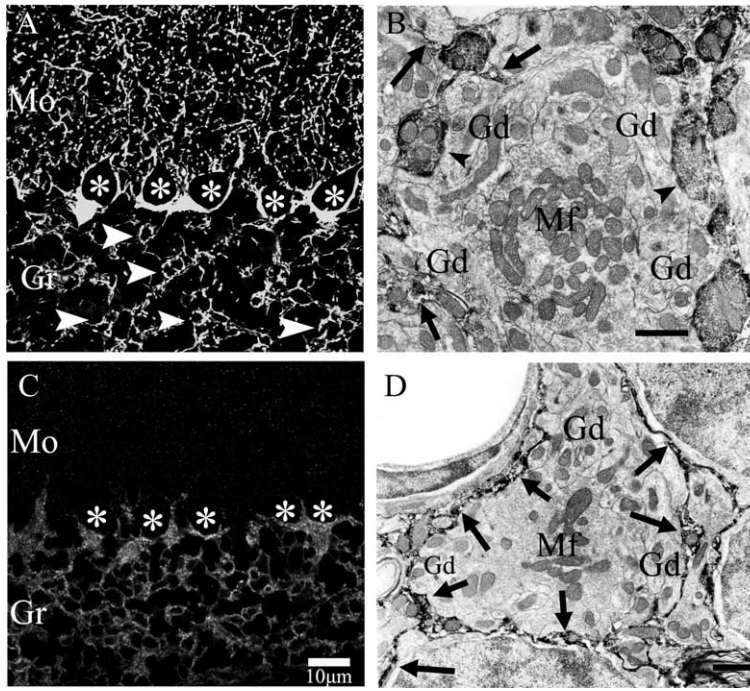

FIG. 3. Immunohistochemical localization of GAT-1 (A, B) and GAT-3 (C, D) in the adult mouse cerebellar cortex. (A and B) GAT-1-immunolabeling is localized at the axon terminals of GABAergic neurons in the molecular (Mo), Purkinje cell, and granular (Gr) layers. In the granular layer, GAT-1-immunolabeling exhibits ring-shaped profiles (white arrowheads) at the synaptic glomeruli in a light micrograph (A). An electron micrograph of the granular layer shows that the immunolabeling is detected at the axon terminals, which contain flat vesicles and form symmetric synapses (black arrows) with granule cell dendrites (Gd) (B). In addition, weak immunolabeling is also observed at the astrocytes (black arrows). (C and D) GAT-3 immunolabeling is detected in the neuropil of Purkinje cell and granular (Gr) layers in the light micrograph (C), and localize at the processes of astrocytes (black arrows) in the electron micrograph (D). Abbreviations and symbols: Mo: molecular layer, Gr: granular layer, asterisk: Purkinje cell body, white arrowhead: synaptic glomerulus, Mf: mossy fiber terminal, Gd: granule cell dendrite, black arrowhead: symmetric synapse, black arrows: immunolabeling in the process of astrocytes. Bar in the EM: 1μm.

III. GABAergic Signaling Before Synaptogenesis

A. Extrasynaptic GABA Release in the Developing Cerebellum

In the developing CNS, GABA appears in GABAergic neurons long before the onset of synaptogenesis (Fairen *et al.*, 1998; Lauder, 1993; Lauder *et al.*, 1986; Van Eden *et al.*, 1989) and its subcellular localization gradually changes during

brain development (Behar *et al.*, 1993; McLaughlin *et al.*, 1975; Takayama and Inoue, 2004b). In the cerebellum, before GABAergic synapses are formed, GABA is distributed throughout the GABAergic neurons, including cell bodies, dendrites, axons, axon varicosities, and growth cones (Takayama and Inoue, 2004a; Takayama and Inoue, 2004c) (see Fig. 3A, 4A). VGAT, which is a membrane protein of GABAergic vesicles and transports cytosolic GABA into the vesicles (Chaudhry *et al.*, 1998; Dumoulin *et al.*, 1999; Fon and Edwards, 2001; Reimer *et al.*, 1998; Takamori *et al.*, 2000), accumulates at the axon varicosities and growth cones where GABAergic synapse are not yet formed (Fig. 3B, C, 4B, C) (Takayama and Inoue, 2004a). This indicates that GABA is distributed throughout GABAergic neurons, and vesicular GABA accumulates to the axon varicosities and growth cones. During synapse formation, GABA becomes confined to the axon terminals, and gradually disappears from axons themselves as well as dendrites. After finishing synapse formation, GABA is almost completely co-localized with VGAT at the synaptic sites where the $GABA_A$ receptor $\alpha 1$ subunit accumulates (Fig. 4D-F). This indicates that most GABA is exclusively localized in the synaptic vesicles within the axon terminals.

Physiological and biochemical studies have demonstrated that the non-vesicular form of GABA is also secreted via the plasma membrane by reverse transporter actions of GATs (see Fig. 4D) (Attwell *et al.*, 1993; Behar *et al.*, 1993; Belhage *et al.*, 1993; Gao and van den Pol, 2000; Jaffe and Vaello, 1988; Taylor *et al.*, 1990; Taylor and Gordon-Weeks, 1991; Varju *et al.*, 2001). In the developing brain, GABA could be released in two ways: exocytosis of GABAergic vesicles, and diacrine of cytosolic GABA via plasma membrane (see Fig. 5D). It was hypothesized that cytosolic GABA might be extrasynaptically released from dendrites, axons, and cell bodies via the plasma membrane by GATs, and GABA in the vesicles might be also extrasynaptically released from axon varicosities and growth cones (Varju *et al.*, 2001).

To clarify which type of release occurs in the developing cerebellum, we examined the changes in distribution of the plasma membrane GABA transporters in the developing cerebellum (Takayama and Inoue, 2005). We could not find GAT-1 or GAT-3 in the dendrites and cell bodies in the developing GABAergic neurons (see Fig. 6). GAT-1 first appears in the granular layer and subsequently in the Purkinje and molecular layers, localizing at axons, varicosities, and terminals (Fig. 6A-F). GAT-3 appears at P10 in the deep part of the granular layer and localized in the processes of astrocytes (Fig. 6F, G). These localizations are the same as those in the adult cerebellum (Durkin *et al.*, 1995; Itouji *et al.*, 1996; Morara *et al.*, 1996; Ribak *et al.*, 1996; Rosina *et al.*, 1999). These results suggest that GABA is synthesized throughout the GABAergic neurons and transported into vesicles but is not released by diacrine. GABA in the vesicles is confined to the axon varicosities and growth cones and released by

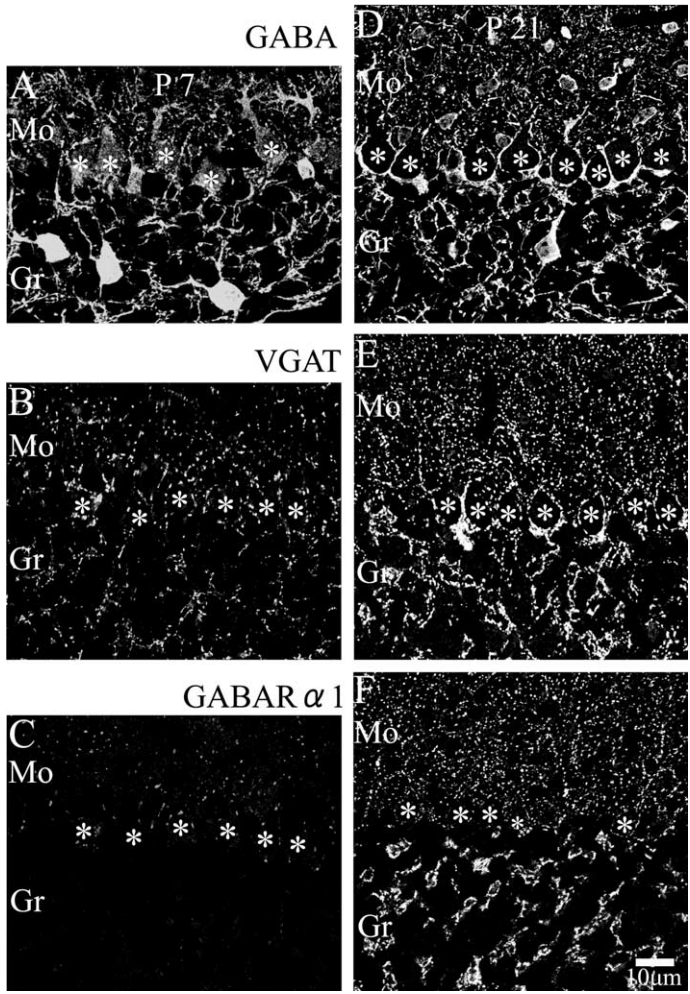

FIG. 4. Immunohistochemical localization of GABA (A, D), VGAT (B, E), and $GABA_A$ receptor α1 subunit (C, F) in the cerebellar cortex at postnatal day 7 (P7) (A-C) and P21 (D- F). At P7, GABA is localized throughout the GABAergic neurons (A). VGAT, a marker of GABAergic vesicles, is confined to the axon varicosities (B). VGAT is often localized at the axons where mature GABAergic synapses, labeled by the immunohistochemistry for $GABA_A$ receptor α1 subunit (C), are not formed in the granular layer (Gr). In contrast, at P21, the majority of GABA is confined to the terminals (D) where VGAT (E) and α1 subunit (F) are localized. Abbreviations and symbols, Mo: molecular layer, Gr: granular layer, asterisk: Purkinje cell body.

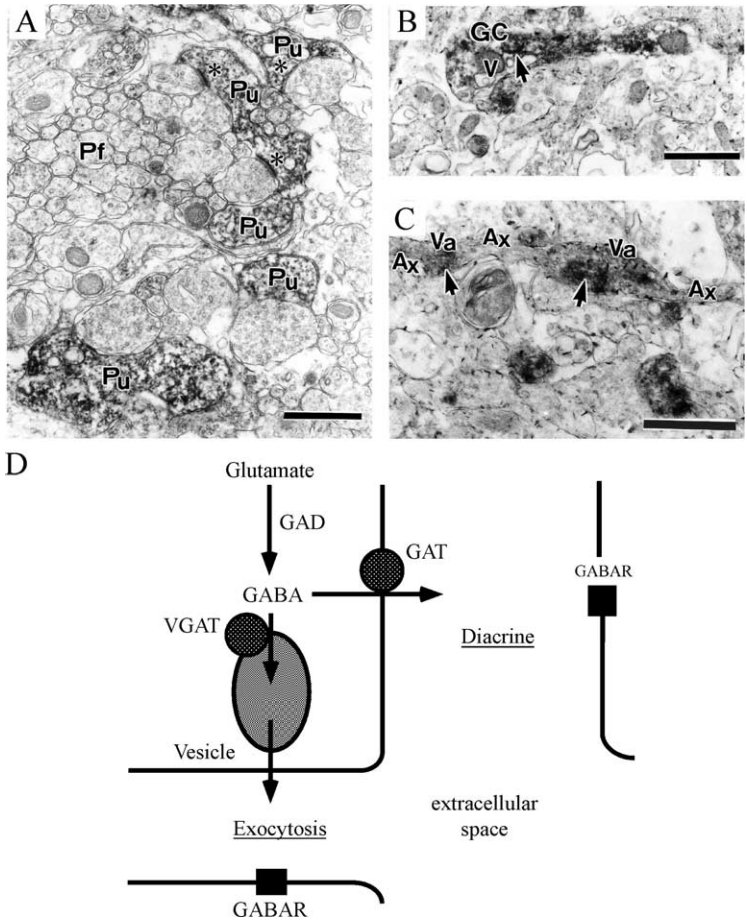

FIG. 5. Electron microscopic localization of GABA (A) and VGAT (B, C) in the immature cerebellum at P5 and the extrasynaptic GABA secretion system (D). (A-C) Electron micrographs of the immunohistochemistry for GABA (A) and VGAT (B, C) in the cerebellum at P5. GABA is distributed throughout the dendrites (A), whereas VGAT is detected at the vesicles (arrowheads) in the growth cone (GC) and axon varicosities (Va). (D) Schematic illustration of the extrasynaptic GABA-release system. Before synapse formation, GABA could be released in two ways: diacrine of cytosolic GABA by plasma membrane GABA transporters (GATs) and exocytosis of GABAergic vesicles. GABA is diffused in the extracellular space and activates GABA receptors (GABAR) on the neighboring neurons. Abbreviations and symbols. Pu: Purkinje cell dendrite, Pf: parallel fiber, asterisk: asymmetric synapse, GC: growth cone, v: vacuole, Ax: axon, Va: axon varicosity, arrowhead: synaptic vesicle, GAD: glutamic acid decarboxylase, VGAT: vesicular GABA transporter, GAT: (plasma membrane) GABA transporter, GABAR: GABA receptor. Bar in the EM=1μm.

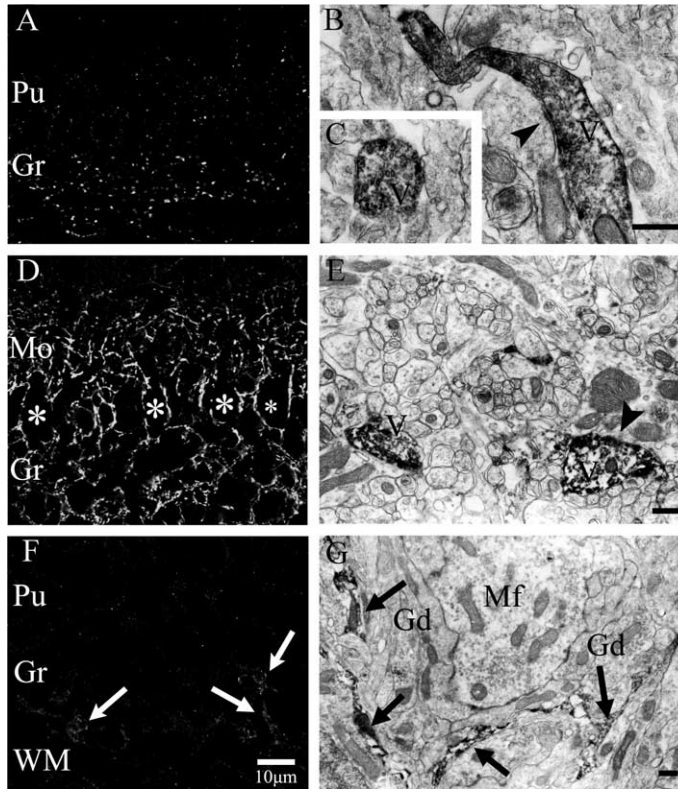

FIG. 6. Developmental expression of GAT-1 (A-E) and GAT-3 (F, G) in the cerebellar cortex. (A-E) Immunohistochemical localization of GAT-1 at P5 (A-C) and P10 (D, E). GAT-1 immunolabeling appears at P5 in the granular layer (Gr) (A), and is localized at the axons and varicosities containing vesicles (V) (B, C). At P10, the immunolabeling is also detected in the molecular (Mo) and Purkinje cell (asterisks) layers and is localized at the axon terminals of stellate cells, which often form symmetric synapses (arrowheads). (F and G) Immunohistochemical localization of GAT3 in the cerebellum at P10. GAT3-immunolabeling (white arrows) appears at P10 in the deep part of the granular layer (Gr) (F), and is localized at the processes of astrocytes (black arrows). Abbreviations and symbols, Mo: molecular layer, Gr: granular layer, asterisk: Purkinje cell body, white arrow: GAT-3 immunolabeling in the granular layer, V: GABAergic vesicle, arrowhead: synapse and synapse-like structure, black arrow: GAT3-positive process of astrocytes, Mf: mossy fiber, Gd: granule cell dendrite. Bar in the EM: 0.5μm.

exocytosis in the developing cerebellum (see Fig. 7A). The exocytosis trigger is unknown. In the mature cerebellum, on the other hand, most GABA is synthesized at the terminals, including varicosities, where synapses are formed and is released at the synapse (Fig. 7B).

Furthermore, the GABA-removing system might shift as shown in Fig. 7C-E. Before synapse formation, GABA is released from axon varicosities and growth

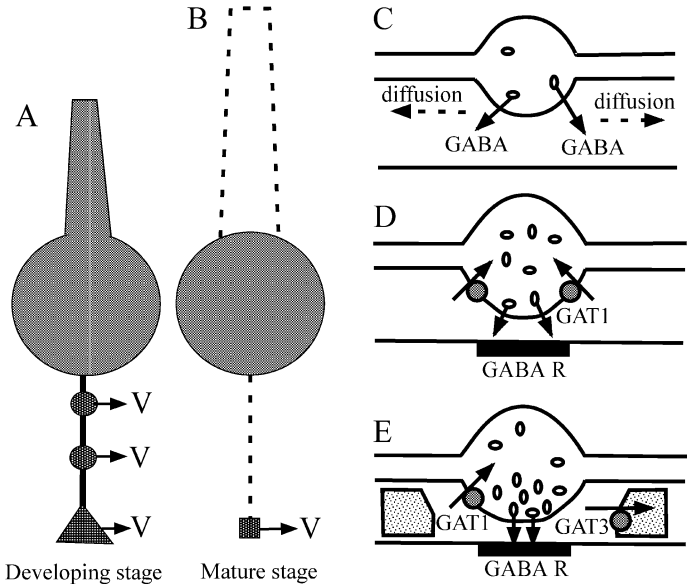

FIG. 7. Developmental changes in GABA-release (A, B) and uptake (C-E) mechanisms in the mouse cerebellum. (A and B) Schematic illustrations of developmental changes in the GABA release system. In the developing cerebellum, GABA is localized throughout the GABAergic neurons and is released by exocytosis (V) from axon varicosities (circle) and growth cones (triangles) (A). In contrast, GABA disappears from dendrites and axons in the mature brain, and is exclusively released synaptically (B). (C-E) Schematic illustrations of developmental changes in the GABA-uptake and reuptake system in the mouse cerebellar cortex. Before synapse formation, GABA is released from axon varicosities and growth cones of GABAergic neurons and disappears by diffusion (C). During synaptogenesis, GAT-1 mediates the reuptake from the extracellular space into the axon and presynapses (D). In the mature cerebellum, GABA is removed from synaptic clefts into the presynapse by GAT-1 and astrocytes by GAT-3 (E). Abbreviations. V: vesicular secretion (exocytosis), GABAR: GABA receptor.

cones of GABAergic neurons and disappears by diffusion (Fig. 6C). During synaptogenesis, GAT-1 mediates the reuptake from the extracellular space into the axons and presynapses (Fig. 7D). Finally, GABA is removed from synaptic clefts into the presynapse by GAT-1 and astrocytes by GAT-3. These results indicate that plasma membrane GATs might not be involved in the diacrine process, but only uptake of GABA from synaptic clefts in the cerebellum.

B. GABAergic Roles in the Developing Brain

During brain development, extrasynaptically released GABA diffuses in the extracellular space and activates GABA receptors on neighboring neurons.

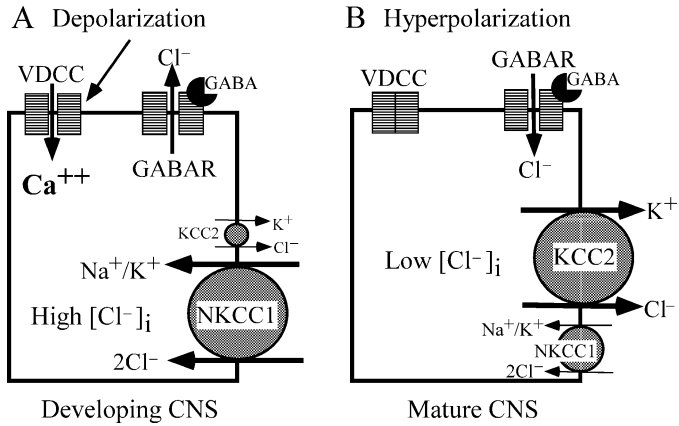

FIG. 8. Schematic illustrations of the developmental changes in GABA actions. (A) In the developing CNS, opening of $GABA_A$ receptors (GABAR) generates efflux of chloride ion (Cl^-) and depolarization of membrane potential, since the intracellular chloride concentration, $[Cl^-]i$, is relatively high due to the dominant action of sodium-potassium-chloride co-transporter 1 (NKCC1). GABA-inducing depolarization activates the voltage dependent calcium channel (VDCC) and mediates calcium influx (Ca^{++}). (B) In the mature CNS, GABA mediates influx of chloride ion (Cl^-), since potassium-chloride co-transporter 2 (KCC2) lowers the intracellular chloride concentration. Influx of chloride ion mediates hyperpolarization of membrane potential.

The activation of $GABA_A$ receptors depolarizes membrane potential, since the Cl^- reversal potential of the neuronal membrane is elevated (see Fig. 8A) (Ben-Ari, 2002; Cherubini *et al.*, 1991; Leinekugel *et al.*, 1999; Owens and Kriegstein, 2002; Perkins and Wong, 1997; Rohrbough and Spitzer, 1996; Serafini *et al.*, 1998). In the developing CNS, Na^+-K^+-$2Cl^-$ co-transporter 1 (NKCC1), which raises the concentration of intracellular chloride ion, $[Cl^-]i$, is predominantly expressed, and elevates the equilibrium potential of Cl^-. Under the high $[Cl^-]i$ condition, the activation of $GABA_A$ receptors generates efflux of chloride ion and depolarization of membrane potential (Fig. 8A). In contrast, K^+-Cl^- co-transporter 2 (KCC2), which lowers the $[Cl^-]i$, becomes a predominant chloride co-transporter in the mature CNS, and GABA induces hyperpolarization of membrane potential and inhibition of excitability (Fig. 8B).

$GABA_A$-receptor-mediated depolarization in the immature CNS activates voltage-dependent Ca^{++} channels (VDCC) and/or N-methyl-D-aspartate (NMDA) type glutamate receptors, and elevates cytosolic Ca^{++} ion (Fig. 8A) (Ben-Ari *et al.*, 1997; Connor *et al.*, 1987; Eilers *et al.*, 2001; Leinekugel *et al.*, 1995; Obrietan and van den Pol, 1996; Reichling *et al.*, 1994; Serafini *et al.*, 1998; Yuste and Katz, 1991). The elevation of cytosolic calcium effects various steps in CNS development such as (1) cell proliferation, (2) cell migration, and (3) neuronal

maturation, including synaptogenesis (Barker *et al.*, 1998; Belhage *et al.*, 1998; Ben-Ari, 2002; Kardos, 1999; McCarthy *et al.*, 2002; Owens and Kriegstein, 2002; Varju *et al.*, 2001). GABA acts as an anti-proliferation molecule, reduces the DNA synthesis in the proliferating precursor cells, and depresses the rate of cellular proliferation by the activation of $GABA_A$ receptors, and other $GABA_A$-receptor related molecules (Haydar *et al.*, 2000; LoTurco *et al.*, 1995). GABA modulates neuronal migration by 'chemotaxis' and 'chemokinesis' at the femtomolar (10^{-15}M) and micromolar (μM) level, respectively (Behar *et al.*, 1996, 1994, 1995). Activation of $GABA_B$ and $GABA_C$ receptors promotes migration out of the proliferating layer, whereas that of the $GABA_A$ receptor slows or almost stops the movement in the cortical plates (Behar *et al.*, 2000). Furthermore, exposure of neurons to GABA or $GABA_A$ receptor agonists induces the synthesis of neuron specific molecules such as neuron-specific enolase (NSE) and neural cell adhesion molecules (NCAMs), enhances the growth rate of neuronal processes, and facilitates synapse formation by inducing the expression and targeting of GABA receptor subunits, which mediate synaptic transmission (Abraham and Schousboe, 1989; Barbin *et al.*, 1993; Belhage *et al.*, 1998; Carlson *et al.*, 1997, 1998; Elster *et al.*, 1995; Gao and van den Pol, 2000; Kim *et al.*, 1993; Meier and Jorgensen, 1986; Meier *et al.*, 1987; Mellor *et al.*, 1998; Mitchell and Redburn, 1996; Moss and Smart, 2001; Spoerri, 1988; Wolff *et al.*, 1978). In the case of interneurons and their networks, GABA might stimulate the expression of neurotrophins, such as brain derived neurotrophic factors (BDNF) and their receptors, and enhance the growth of neurons and synapses (Berninger *et al.*, 1995; Marty *et al.*, 1996; Rico *et al.*, 2002; Vicario-Abejon *et al.*, 1998).

In the cerebellum, the above change in the chloride ion concentration system could not be clarified (Eilers *et al.*, 2001; Kanaka *et al.*, 2001; Lu *et al.*, 1999; Mikawa *et al.*, 2002; Williams *et al.*, 1999). However, GABA could be also involved in morphogenesis in the cerebellum since GABA elevates the Ca^{++} ion concentration in the Purkinje and granule cells during the first two postnatal weeks (Connor *et al.*, 1987; Eilers *et al.*, 2001).

C. $GABA_A$ Receptor Expression in the Developing Cerebellum

In the CNS, the subunit compositions of $GABA_A$ receptors drastically change during brain development (Ben-Ari *et al.*, 1997; Connor *et al.*, 1987; Eilers *et al.*, 2001; Leinekugel *et al.*, 1995; Obrietan and van den Pol, 1996; Reichling *et al.*, 1994; Serafini *et al.*, 1998; Yuste and Katz, 1991). We focused on the α subunits, which may mainly reflect the functional diversity of the $GABA_A$ receptors (Kardos, 1999; Luddens *et al.*, 1990; Macdonald and Olsen, 1994; Olsen and Tobin, 1990; Pritchett *et al.*, 1989; Sieghart, 1995), and investigated the

TABLE I
CHANGES IN EXPRESSION OF THE PREDOMINANT α SUBUNITS OF THE $GABA_A$ RECEPTORS IN THE CEREBELLAR CORTICAL NEURONS

	Proliferating stage	Migrating and differentiating stage	Matured stage
Purkinje cells	negative	$\alpha 3$ subunit	$\alpha 1$ subunit
Granule cells	negative	$\alpha 2$ subunit	$\alpha 1$ and $\alpha 6$ subunits

developmental changes in expression and localization of the $GABA_A$ receptor α subunits in the cerebellum (see Table I) (Takayama and Inoue, 2004c; Takayama and Inoue, 2004d). Proliferating cells in the ventricular zone adjacent to the fourth ventricle and the upper half of the external granular layer expressed no α subunits. Since at least one α subunit is essential for functional $GABA_A$ receptors (McKernan and Whiting, 1996; Pritchett *et al.*, 1989; Sieghart, 1995; Sieghart *et al.*, 1999), receptor activity is absent in the proliferating zone. After finishing cell proliferation, cerebellar neurons start to express the functional $GABA_A$ receptors. Differentiating Purkinje cells express the $\alpha 3$ subunit, migrating and maturating granule cells express the $\alpha 2$ subunit, and both subunits disappear from the cerebellar cortex after synapse formation finishes (Takayama and Inoue, 2004d). In addition, the $\beta 3$, $\gamma 1$, and $\gamma 3$ subunits are also abundantly expressed in the developing cerebellum (Laurie *et al.*, 1992b). These results suggest that extrasynaptically released GABA activates $GABA_A$ receptors consisting of the restricted subunits, and may be involved in the regulation of proliferation, neuronal migration, and maturation in the cerebellum.

D. CONCLUSION FOR THIS SECTION

Before synapse formation, GABA is synthesized throughout the GABAergic neurons, transported into GABAergic vesicles at axon varicosities and growth cones, extrasynaptically released by exocytosis, and diffused within the extracellular space. Released GABA activates GABA receptors consisting of $\alpha 2/3$, $\beta 3$, $\gamma 1/3$ subunits on the neighboring neurons, mediates the depolarization of membrane potential, and induces various types of morphogenesis.

IV. Formation of GABAergic Synapses

Synapse formation is considered to be a multi-step process (Cherubini and Conti, 2001; Moss and Smart, 2001; Vaughn, 1989). While exploring their

environment, axonal growth cones lead elongating axons to their appropriate targets and make contact with dendrites and cell bodies of target neurons. Initial contact is followed by the establishment of stable synapses. In the presynapse, synaptic vesicles accumulate to the nerve terminals and dock near the active zone. In the postsynapse, $GABA_A$ receptors which mediate inhibitory synaptic transmission are targeted to and clustered at an appropriate synaptic site opposite the GABA-releasing site. At the same time, $GABA_A$ receptors, which are involved in brain morphogenesis, disappear from postsynaptic neurons (Takayama and Inoue, 2004b,d).

A. Development of GABAergic Synapses in the Cerebellar Cortex

In the mouse cerebellar cortex, the $GABA_A$ receptor $\alpha 1$ subunit protein, which is an essential subunit of mature $GABA_A$ receptors in Purkinje cells (Laurie *et al.*, 1992a; Persohn *et al.*, 1992), appears at P5 (Takayama and Inoue, 2004c), and symmetric synapses are detected between GAD-positive terminals and Purkinje cell dendrites at the same day (Takayama, 2005). In the mouse granular layer, on the other hand, the $\alpha 1$ and $\alpha 6$ subunit proteins, which are essential subunits for the mature $GABA_A$ receptors in the granule cells, appear in deep part at P7, and symmetric synapses are clearly discernible at P10 between GAD-positive terminals and granule cell dendrites in the synaptic glomeruli.

These results indicate that in the cerebellum excitatory synapses appear prior to the inhibitory synapses, and GABAergic synapses start to be formed on the Purkinje cell dendrites during the first postnatal week, and granule cell dendrites during the second postnatal week. The number of GABAergic synapses increase dramatically in all layers during the second and third postnatal weeks (Altman and Bayer, 1997; Jakab and Hamori, 1988; Larramendi, 1969; Takayama and Inoue, 2004c).

B. Target Determination

It is not fully understood how GABAergic neurons search, recognize, and determine their target neurons. To reveal how GABAergic neurons determine their targets and form synapses, we employed cerebellar mutant mice and examined the specificity of neuron-to-neuron connection in the mutant cerebellum. In the normal cerebellar cortex, five major types of neurons innervate distinct types of target neurons (Fig. 1B) (Ito, 1984; Llinas and Hillmann, 1969; Palay and Chan-Palay, 1974). The specific innervation patterns, however, are not preserved in the abnormal environment of the reeler and weaver cerebellum (see Fig. 9). Golgi cells directly innervate to Purkinje cells in the central mass of the

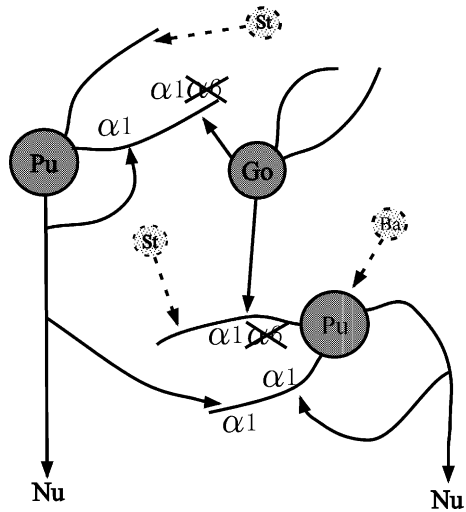

FIG. 9. Schematic illustrations of the abnormalities in the GABAergic inputs in the reeler cerebellum. In the central cerebellar mass of the reeler cerebellum, Purkinje cells (Pu) receive inhibitory inputs (arrows) from Golgi cells (Go) instead of stellate (st) and basket (ba) cells. $GABA_A$ receptors containing only the $\alpha 1$ subunit ($\alpha 1$), but not the remaining five α subunits, are localized at the GABAergic synapses on the Purkinje cells, although Golgi cells innervate them. In addition, GABAergic input from the Purkinje cell axon collaterals increased markedly. Abbreviations; Pu: Purkinje cell, Go: Golgi cell, St: stellate cell, Ba: basket cell, Nu: cerebellar nucleus.

reeler cerebellum and in the cortex of the weaver cerebellum (Caviness and Rakic, 1978; Mariani *et al.*, 1977; Rakic, 1976; Sotelo and Privat, 1978; Takayama, 1994; Wilson *et al.*, 1981). In both regions, granule cells are scarce or absent. Thus, Golgi cell axons form synapses with neighboring neurons instead of granule cells. This result indicates that targets of Golgi cells are not genetically and strictly determined, but are influenced by the environment, and that targets of GABAergic neurons plastically alter according to the environment.

C. CHANGE IN SUBUNIT COMPOSITIONS

As shown in Table I, expression of the $GABA_A$ receptor α subunits in the cerebellum developmentally changes especially during GABAergic synapse formation. While expression of the $\alpha 2$ and $\alpha 3$ subunits is decreasing, the $\alpha 1$ and $\alpha 6$ subunits appear and increase their expression (Laurie *et al.*, 1992b; Mellor *et al.*, 1998; Takayama and Inoue, 2004c,d; Tia *et al.*, 1996). Therefore, the α subunits in the $GABA_A$ receptors shift from the $\alpha 2$ and $\alpha 3$ subunits to the $\alpha 1$ and $\alpha 6$

subunits during cerebellar development. This result indicates that two pieces of evidence, the disappearance of subunits involved in morphogenesis, and the appearance of subunits which mediate inhibitory synaptic transmission, are crucial for GABAergic synapse formation.

To test the mechanism underlying the change in subunit compositions, we investigated its relationship with neuronal maturation, including migration, axonal, and dendritic extension, and formation of excitatory and inhibitory synapses using reeler mutant mice. In the reeler cerebellum, maturation of malpositioned Purkinje cells is assumed to be arrested in terms of the synaptic architecture and dendritic arborization (Caviness and Rakic, 1978; Mariani *et al.*, 1977; Rakic, 1976; Sotelo and Privat, 1978; Takayama, 1994; Wilson *et al.*, 1981). Parallel fibers and axons from stellate and basket cells do not innervate the Purkinje cells in the central cerebellar mass. Moreover, multiple innervations from climbing fibers remain in the adult reeler cerebellum. Instead, Purkinje cells directly form synapses with mossy fibers and Golgi cell axons. Dendrites of Purkinje cells are poorly developed and extend almost randomly. The $\alpha 3$ subunit, however, is almost negative, as in the normal mature cerebellum (Fig. 10E, F), and malpositioned Purkinje cells abundantly express the $\alpha 1$ subunit (Fig. 10A, B) (Frostholm *et al.*, 1991; Takayama and Inoue, 2003). These results indicate that developmental change in subunit composition is independent of neuronal maturation such as settling in the normal neuronal position, maturation of excitatory networks. Absence of normal inhibitory synapses with stellate and basket cell axons and heterologous input from Golgi cells do not affect the developmental changes in subunit composition. Previous in vitro studies have indicated that GABAergic stimulation induces low-affinity type GABA receptor expression, which is involved in inhibitory synaptic transmission (Belhage *et al.*, 1998; Belhage *et al.*, 1986; Carlson *et al.*, 1997, 1998; Elster *et al.*, 1995; Gao and Fritschy, 1995; Kim *et al.*, 1993; Meier *et al.*, 1984; Mellor *et al.*, 1998; Raetzman and Siegel, 1999; Schousboe, 1999). The change in subunit composition simultaneously occurred during GABAergic synaptogenesis (Table I). These results suggest that innervation of GABAergic fibers may be important for the change in subunit composition, even if the synapses are heterologous and ectopic, and GABAergic innervation might initiate and/or accelerate the changes in subunit composition.

D. Specific Subunit Expression

In the CNS, distinct types of subunits are expressed at distinct synapses (Fig. 1B) (Laurie *et al.*, 1992a; Persohn *et al.*, 1992; Wisden *et al.*, 1992). In the normal cerebellum, GABAergic transmission between stellate cell axons and Purkinje cell dendrites is mediated by $GABA_A$ receptors containing only the $\alpha 1$

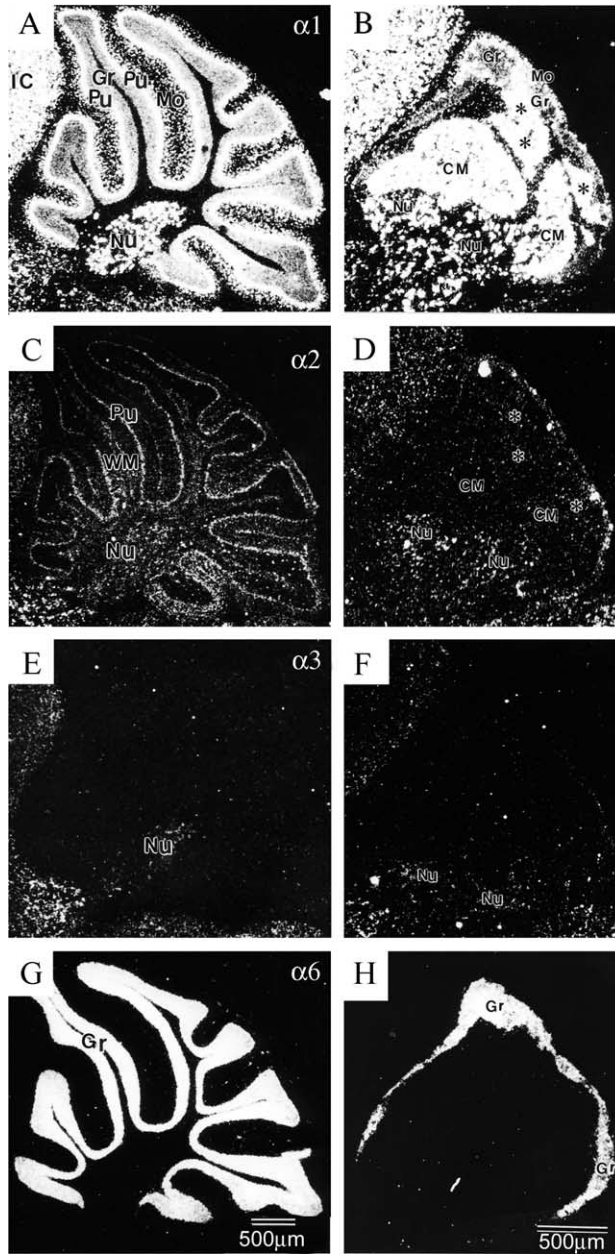

FIG. 10. Distinct expression of the $GABA_A$ receptor $\alpha 1$ (A, B), $\alpha 2$ (C, D), $\alpha 3$ (E, F), and $\alpha 6$ (G, H) subunits in the normal (A, C, E, G), and reeler (B, D, F, H) cerebella. The specific expression of α subunit mRNAs in each neuronal type was preserved in the reeler cerebellum. Furthermore,

subunit, but not the remaining five α subunits (Fig. 1B) (Laurie *et al.*, 1992a; Persohn *et al.*, 1992; Wisden *et al.*, 1992, 1996). In contrast, inhibitory transmission between Golgi cell axons and granule cell dendrites is mediated by $GABA_A$ receptors containing both $\alpha 1$ and $\alpha 6$ subunits.

To test the relationship between types of presynapse and subunits in the postsynapse, we examined the expression of $GABA_A$ receptor α subunits in the reeler cerebellum. In the central cerebellar mass of the reeler cerebellum, Purkinje cells directly form synapses with Golgi cell axons (Fig. 9) (Caviness and Rakic, 1978; Mariani *et al.*, 1977; Rakic, 1976; Sotelo and Privat, 1978; Takayama, 1994; Wilson *et al.*, 1981). If presynaptic neurons determine the type of receptor subunits in postsynaptic neurons, GABAergic innervation from Golgi cells would induce Purkinje cells to express the $\alpha 6$ subunit in the central cerebellar mass. Nevertheless, Purkinje cells in the central cerebellar mass do not express the $\alpha 6$ or $\alpha 2$ subunits (Fig. 10C, D, G, H) (Takayama and Inoue, 2003). This result indicates that Golgi cell innervation does not induce expression of the $\alpha 6$ subunit in Purkinje cells, and suggests that postsynaptic self-autonomous mechanisms determine the types of subunits (Fig. 9).

E. Synaptic Targeting and Clustering of $GABA_A$ Receptor Proteins

Synaptic targeting and clustering of $GABA_A$ receptors are mediated by the interaction of the subunit proteins with the subsynaptic cytoskeleton, and it is thought that the diversity of subunits in the $GABA_A$ receptors is important for subcellular localization (Barnes, 2000; Moss and Smart, 2001). Most single subunits are retained within the endoplasmic reticulum (Connolly *et al.*, 1996; Gorrie *et al.*, 1997; Taylor *et al.*, 2000). Specific subunits such as the $\gamma 2$ subunit can lead the assembled $GABA_A$ receptors to the cell surface and synaptic site, clustering (Connolly *et al.*, 1999) in conjunction with a range of diverse anchoring protein molecules such as gephyrin (Craig *et al.*, 1996; Essrich *et al.*, 1998; Kneussel *et al.*, 1999; Sassoe-Pognetto and Fritschy, 2000), $GABA_A$-receptor associated protein (GABARAP) (Wang *et al.*, 1999), microtubule-associated proteins, transporters, protein kinases, and so on (Moss and Smart, 2001). Furthermore, anchoring proteins such as gephyrin and GABARAP are also involved in clustering of receptor proteins (Barnes, 2000; Moss and Smart, 2001).

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abnormal expression of α subunits was not detected, although GABAergic networks were altered and neuronal maturation is severely disturbed. Abbreviations: IC: inferior colliculus, Mo: molecular layer, Pu: Purkinje cell layer, Gr: granular layer, Nu: cerebellar nucleus, WM: white matter, asterisks: central cerebellar mass beneath the granular layer, CM: central cerebella mass under the white matter.

F. Activity-Dependent Synaptic Remodeling

Recent investigations revealed that GABAergic synapses are remodeled by the change in GABAergic input in auditory systems during the critical period (Kandler, 2004; Kapfer *et al.*, 2002; Kim and Kandler, 2003). Auditory experience guides subcellular localization of receptor proteins (Kapfer *et al.*, 2002), induces functional and structural elimination of inhibitory synapses during the establishment of precise topography in the GABAergic/glycinergic pathway (Kim and Kandler, 2003), and mediates aural dominance bands in the inferior colliculus (Gabriele *et al.*, 2000). In the cerebellum, the activity-dependent remodeling of GABAergic synapses has not yet been clarified, but could play roles in the formation and maturation of GABAergic synapses and networks.

G. Conclusion for this Section

GABAergic axons determine their target neurons under the influence of environmental conditions. During the formation of GABAergic synapses, axon varicosities and growth cones which contains GABAergic vesicles give rise to presynapse. GABA-release could induce the maturation of postsynapse, including expression of the mature type receptor subunits, disappearance of immature type subunits, and targeting of subunit proteins. At the postsynapse, genetically determined subunits are expressed.

V. Functions of GABAergic Synapses in the Cerebellum

The cerebellum is closely involved in learning motor skills (Ito, 1984; Llinas and Walton, 1990). GABAergic input might play important roles in cerebellar functions since GABAergic neurons regulate the neuronal activity of Purkinje cells and granule cells which organize the major stream of neural circuitry in the cerebellar cortex. Neuroanatomical analysis of the cerebellar local circuit suggests that GABAergic neurons play a role in lateral inhibition and negative feedback mechanisms on the Purkinje and granule cells. Furthermore, elimination of GABAergic input from the Golgi cells in the cerebellar granular layer caused overexcitation of granule cells resulting in severe ataxia during the acute phase (Watanabe *et al.*, 1998). Therefore, GABAergic input plays a role in the regulation of glutamatergic hyperexcitation and could be involved in motor coordination.

In addition, neuroimaging and biochemical studies indicate a dysfunction in the GABAergic system in the cerebellum of autistic patients (Dhossche, 2004;

Fatemi *et al.*, 2002). This result suggests that the GABAergic network in the cerebellum might be involved in not only motor function, but also higher brain functions.

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INSIGHTS INTO GABA FUNCTIONS IN THE DEVELOPING CEREBELLUM

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During development, GABA, the main inhibitory neurotransmitter of the nervous system is excitatory and elicits calcium-dependent trophic effects in developing neurons. Through depolarization of immature cerebellar neurons, GABA increases intracellular calcium and the phosphorylation of MAPK and CaMKII. Other depolarizing stimuli, such as exposure to 25 mM KCl, accelerate the switch of GABA from depolarizing to hyperpolarizing. Exposure to GABA and muscimol increases proliferation of immature granule cells. In addition, GABA increases the size of the dendritic arbor in early postmitotic neurons. This chapter outlines the role of GABA in the development of cerebellar granule neurons, and other central neurons, and reviews evidence that GABA and other depolarizing stimuli play a crucial role in the early development of the cerebellum.

I. Introduction

The GABAergic system is affected in many neuropathological conditions including autism (Fatemi *et al.*, 2002b). Morphological alterations, consisting of loss of granule and atrophy as well as loss of Purkinje cells (Fatemi *et al.*, 2002a;

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Ritvo *et al.*, 1986), have been reported in the cerebellum of autistic patients. This article reviews a series of studies outlining the role of GABA in the development of the central nervous system and particularly in the development of the cerebellum. These studies provide evidence supporting the crucial role for GABA during development, the knowledge of which may be fundamental to a better understanding of the pathogenesis of autism and related diseases.

II. The Adult Cerebellum

The mammalian cerebellar cortex is an anatomically well-defined structure with a particular laminated cytoarchitecture. The cerebellum is composed of a limited number of neuronal cell types, identifiable by their position and size, and includes granule, Purkinje, Golgi, basket, and stellate neurons (Palay and Chan-Palay, 1974).

Purkinje cells are inhibitory interneurons organized in a cell layer between the granule and the molecular layers of the cerebellum. They constitute the output path of the cerebellum, and each of them receives input from just one climbing fiber axon, which originates in the inferior olive. The climbing fiber forms a very strong synapse onto the Purkinje cell, with each presynaptic spike triggering a postsynaptic spike. Climbing fibers may serve a special function other than ordinary signal transmission; an instructive signal that regulates the strength of parallel fiber–Purkinje cell synapses. Two possible actions were postulated for the synapses between climbing fibers and Purkinje cells: they may act as positive reinforcers, strengthening parallel fiber synapses, and/or as error signals, serving to weaken synapses (see review by Boyden *et al.*, 2004). Cerebellar granule cells are excitatory interneurons and their axons form the parallel fibers that project to Purkinje cells but also form synapse contacts with cells in the molecular layer. Cerebellar granule cells receive inputs from the mossy fibers coming from the vestibular nuclei. The Golgi cells are inhibitory interneurons of the cerebellum that have their cell bodies located in the granule layer. Golgi cells receive inputs from, and project their inhibitory output to granule cells, constituting a negative feedback circuit. Stellate cells are small cells lying in the outer two thirds of the molecular layer; basket cells are located in the inner third, in close proximity to the Purkinje cell layer. The basket and stellate cells receive excitatory inputs from the parallel fibers of the granule cells and the climbing fibers and direct their output by inhibiting Purkinje cells and granule cells. Anatomical and physiological studies indicate that molecular layer interneurons lie on the activated parallel fiber beam (Pouzat and Hestrin, 1997) suggesting that a Purkinje cell may be both directly excited and then inhibited via molecular layer interneurons activated by the same set of active parallel fibers (feed-forward inhibition).

The cerebellum is implicated in motor learning, a function likely accomplished by a number of plasticity mechanisms including the long-term depression (LTD) at synapses between parallel fibers and Purkinje cells. LTD is induced when parallel and climbing fiber activities occur simultaneously, whereas LTP is induced when parallel fiber activity and not climbing fiber activity occur (see review by Boyden *et al.*, 2004).

III. The Developing Cerebellum

In the rodent, all cerebellar neurons generate between the end of the second week of gestation and the first week of life. The genesis of Purkinje cells ends at the beginning of the first week of life but its dendritic tree continues to grow until day 21. The granule cells complete their genesis between 7 and 9 days after birth. These proliferating cerebellar granule cell precursors and young differentiating granule neurons migrate from the external granule layer to the internal granule layer, their final destination, guided by Bergman glial fibers (Komuro and Rakic, 1998). Basket and stellate cells reach the molecular layer postnatally, where they are innervated by granule cell axons (parallel fibers) that provide glutamatergic input (Eccles *et al.*, 1967; Palay and Chan-Palay, 1974). The synaptogenesis between parallel fibers and Purkinje and/or basket and stellate cells located in the molecular layer takes place between the first and third weeks of life (Eccles, 1967).

IV. $GABA_A$ Receptors and the Developing Cerebellum

The cerebellum is widely populated with amino acids receptors, mainly GABA and glutamate receptors, making it a very interesting preparation to study amino acids receptor pharmacology (Carlson *et al.*, 1998). The $GABA_A$ receptor system consists of 19 different subunit genes (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ρ_{1-3}, ε, π, and θ) that encode the subunits of pentameric $GABA_A$ receptors (reviewed by Sieghart *et al.*, 1999; Lüscher and Keller, 2004). In granule cells from adult cerebella, high levels of α_1, α_6, β_2, β_3, γ_2, and δ mRNAs are found in comparison to low levels of the mRNAs for the α_4, β_1, and γ_3 subunits (Laurie *et al.*, 1992a). The α_6 subunit has a unique distribution in the brain, in that it is almost exclusively found in cerebellar granule cells (Laurie *et al.*, 1992a). Immunocytochemical studies have likewise shown a dense population of α_1, $\beta_{2/3}$, and δ subunits and moderate γ immunoreactivity in the granule cell layer (see review by Carlson *et al.*, 1998).

$GABA_A$ receptors subunits are expressed in the cerebellum at early stages of perinatal development and the expression pattern varies according with the stage of neuronal maturation (Laurie *et al.*, 1992b). For example, dividing precursor and pre-migratory post-mitotic cells express transcripts encoding the $GABA_A$ receptor α_2, α_3, β_3, γ_1, and γ_2 subunits (Laurie *et al.*, 1992b). Later, α_2, α_3, and γ_1 are down-regulated and replaced by the adult complement that consists predominantly of α_1, α_6, β_2, β_3, γ_2, and δ (Laurie *et al.*, 1992a,b; Thompson and Stephenson, 1994). The α_1, α_6, and δ genes are expressed only in post-migratory cerebellar granule neurons (Kuhar *et al.*, 1993; Laurie *et al.*, 1992b). Electrophysiological recording in the developing cerebellum reveals that a sequential expression of α subunits underlies changes in functional efficacy of the GABAergic network (Ortinski *et al.*, 2004; Vicini *et al.*, 2001). A 50% decrease in the decay time of miniature inhibitory postsynaptic currents (mIPSCs) between cultured cerebellar granule neurons grown for 6 days and those grown for 12 days was paralleled by the decrease of α_2 and α_3 subunits, the increase of α_1 and α_6 subunits expression, and changes in the action of selective α-subunit modulators (Ortinski *et al.*, 2004). How these electrophysiological changes correlate with developmental processes triggered by GABA in the immature cerebellum remains to be established.

V. Depolarization and Neurotrophins Are Trophic Signals

Neurotrophins are a family of peptide growth factors, including neurotrophin-3 (NT-3), NT-4/5, nerve growth factor, and brain-derived neurotrophic factor (BDNF). They play important roles in the development and maturation of the nervous system and support survival of mature neurons (Kaplan and Miller, 2000). Neurotrophins exert their trophic effects by binding to the tyrosine kinase (Trk) receptor family subtype (Chao, 1992) and triggering downstream activation of *ras*-MAP kinase (Ras-mitogen-activated protein kinase, erk) and the phosphatidylinositol-3-OH (PI3K)-protein kinase B (PKB-Akt) pathways (see review by Kaplan and Miller, 2000). Neural activity can mimic the trophic effect induced by neurotrophins promoting the differentiation and survival of central and peripheral neurons (Borodinsky *et al.*, 2002; Franklin *et al.*, 1995; Hansen *et al.*, 2001; Kaplan and Miller, 2000; Vaillant *et al.*, 2002). Intracellular cascades activated by neurotrophins are also activated by neural activity-induced calcium fluctuations (Dolmetsch *et al.*, 2001; Rosen *et al.*, 1994; Vaillant *et al.*, 1999). Furthermore, intracellular calcium fluctuations triggered by neural activity modulate gene expression, as do neurotrophins (Bradley and Finkbeiner, 2002).

The neurotrophins NT3/4 and BDNF are well known neurotrophic factors and their receptors are expressed in the developing cerebellum of humans

(Quartu *et al.*, 2003) and rodents (Gao *et al.*, 1995). In the rodent cerebellum neurotrophins play a critical role in the differentiation and survival of developing basket and stellate cells (Fiszman *et al.*, 2005; Spatkowski and Schilling, 2003) and in granule cell survival (Gao *et al.*, 1995; Shalizi *et al.*, 2003).

Ionotropic GABA and glutamate receptors are expressed at relatively early stages of brain development (Laurie *et al.*, 1992b; Monyer *et al.*, 1994) and functional voltage-gated calcium channels (VGCC) are expressed even in neuroblasts (Fiszman *et al.*, 1990, 1993). Studies performed in different regions of the developing central nervous system (CNS) of the rodent reported that GABA, the main inhibitory neurotransmitter in the adult CNS, triggers depolarizing responses through $GABA_A$ receptor activation (Ben-Ari, 2002) before the appearance of glutamatergic neurotransmission takes place (Ben-Ari *et al.*, 1997). During the development of the cerebellum, cell migration requires fluctuations of intracellular calcium triggered by the activation of VGCC or NMDA receptors (Komuro and Rakic, 1992, 1993). In the embryonic cortex, the early presence of GABA and $GABA_A$ receptors in the marginal zone and subplate (Meinecke and Rakic, 1992) may mediate GABA-induced chemotaxis and chemokinesis. These $GABA_A$-mediated effects are calcium-dependent (Behar *et al.*, 1996).

In hippocampal neurons in culture, exposure to glutamate increases calcium and neurite outgrowth (Wilson *et al.*, 2000) and a glutamate-induced regulation of dendritic growth mediated by AMPA/Kainate receptors was described in motor neurons (Metzger *et al.*, 1998). In addition, VGCC activation increases neurite outgrowth in primary cultured sympathetic (Vaillant *et al.*, 2002), cerebellar granule (Borodinsky *et al.*, 2002), and cortical neurons (Redmond *et al.*, 2002).

Depolarizing responses can increase neuronal survival in the absence of added trophic factors (Chalazonitis and Fischbach, 1980; Hansen *et al.*, 2001; Wakade *et al.*, 1983). Activation of sodium channels promotes survival of *substantia nigra* neurons (Franklin *et al.*, 1995) and sympathetic and spiral ganglion neurons respond positively to the survival promoting effect of high potassium, being in all cases due to an increase in intracellular calcium (Hansen *et al.*, 2001; Vaillant *et al.*, 1999). Furthermore, depolarization may promote survival by stimulating the synthesis and release of BDNF, as has been demonstrated in developing cortical neurons treated with high potassium (Ghosh *et al.*, 1994) and a cAMP-dependent pathway mediates the survival-promoting effects of depolarization in retinal ganglion cells (Meyer-Franke *et al.*, 1995). On the contrary, the survival-promoting effect of high potassium on cerebellar granule neurons is not due to an increased availability of BDNF (Armanino *et al.*, 2005).

A great amount of evidence points to the fact that that survival of cerebellar cells is improved in depolarizing culture conditions mimicked by exposure to 25 mM KCl or to 50–100 micromolar concentrations of NMDA (Armanino *et al.*, 2005; Balázs *et al.*, 1989; Borodinsky *et al.*, 2002; Fiszman *et al.*, 2005; Gallo *et al.*, 1987). The effects of NMDA and high potassium are blocked by

CaMKII blockers (Borodinsky *et al.*, 2002; Hack *et al.*, 1993) and VGCC blockers (Borodinsky *et al.*, 2002; Gallo *et al.*, 1987). Treatment of the cultures with 50 μM kainate during the first week *in vitro* leads to an increased survival of cerebellar granule cells and VGCC are involved (Balázs *et al.*, 1990a,b). However, in the presence of the same kainate concentration stellate, basket, and Golgi cells are eliminated (Damgaard *et al.*, 1996; Drejer and Schousboe, 1989), suggesting that different mechanisms are involved in the survival of granule cells and inhibitory interneurons.

VI. Establishing an *In Vitro* Model to Study the Developing Cerebellar Granule Neurons

Since granule cells constitute the vast majority of cells of the cerebellum and their mitosis takes place later than the other phenotypes, it is easy to establish an almost pure primary granule cells culture (Messer, 1977; Schousboe *et al.*, 1989). The standard procedure to culture cerebellar granule cells is to obtain and plate a cell suspension from 7- to 8-day-old mice or rats. Microarray analyses of cultured granule neurons and developing cerebellar tissue revealed that the time-course of gene expression in cultured granule neurons resembles that observed *in vivo*, confirming that these cells are a good model for the study of developmentally regulated events that take place in the intact cerebellum (Diaz *et al.*, 2002).

Taking advantage of cerebellar granule cell cultures we studied the role of neural activity on the development of cerebellar granule neurons. We focused on the neurogenesis and differentiation of cerebellar granule neurons. In addition, we were interested in the mechanism by which depolarizing responses affect the survival of early postmitotic neurons, since it is well known that these cells depend on depolarization for their survival (Gallo *et al.*, 1987). We studied the characteristics of cerebellar granule cells at different times *in vitro* and, starting from the first hours after plating, determined the time course of the proliferation of these cells by carrying out [^{3}H]-thymidine incorporation assays. We measured the complexity of the dendritic morphology by staining early postmitotic neurons with tetanus toxin fragment C labeling (a postmitotic neuronal marker, Neale *et al.*, 1993). We quantified the fractal dimension in the cells labeled with Fragment C, grown at very low density (Borodinsky and Fiszman, 2001). The long term survival of these cells was determined by measuring the number of cells after one week *in vitro* (DIV7).

During the first 24 hours *in vitro*, immature cerebellar granule cells looked rounded with very few neurites and were not stained with tetanus toxin fragment C (Borodinsky and Fiszman, 1998). Measuring [^{3}H]-thymidine incorporation in these cells revealed that they were capable of proliferating during the first

24 hours after plating (DIV1) and to a lesser extent at DIV2, reaching almost blank levels at 48 hours after plating. At the same time that a decrease in proliferation was seen (DIV2), the majority of cells became positive for tetanus toxin fragment C and acquired morphological features of differentiated phenotypes (Borodinsky and Fiszman, 1998). We therefore used the following schedule; we explored the development of cerebellar granule cells by determining [^{3}H]-thymidine incorporation at DIV1, and differentiation was determined at DIV3. Furthermore, we determined long-term survival of these cells at DIV7. $GABA_A$ agonists, high potassium, and specific blockers were added two hours after plating for the proliferation assays and at 48 hours after plating for differentiation and survival assays. GABA and muscimol (a selective $GABA_A$ agonist) effects were compared to those seen with depolarizing potassium concentrations (25 mM KCl).

VII. GABA-Mediated Depolarization and Calcium Increases

GABA, the main inhibitory neurotransmitter in the adult CNS, is excitatory during development (Ben-Ari *et al.*, 1989; Fiszman *et al.*, 1990, 1993; Mandler *et al.*, 1990; Obata *et al.*, 1978). Due to the depolarizing effect described for GABA and because $GABA_A$ receptors are already present prenatally, before synaptogenesis takes place (Laurie *et al.*, 1992b), a paracrine trophic role of GABA during embryogenesis and early postnatal life was postulated (see reviews by Cherubini *et al.*, 1991; Nguyen *et al.*, 2001; Ben-Ari, 2002; Fiszman and Schousboe, 2004). Depolarization of the cell membrane by GABA in developing neurons appears to be mediated by a reversal of the chloride gradient prior to maturation (Cherubini *et al.*, 1991; Mueller *et al.*, 1984; Owens *et al.*, 1996; Rohrbough *et al.*, 1996). As a consequence of GABA-mediated depolarization, an activation of VGCC takes place with the concomitant intracellular calcium increases, as reported in the hippocampus (Gaiarsa *et al.*, 1995), cerebral cortical neurons (Yuste *et al.*, 1991), postnatal cerebellar granule cells explants (Connor *et al.*, 1987), hypothalamus (Obrietan and van den Pol, 1995), and oligodendrocytes (Kirchhoff and Kettenmann, 1992). The physiological timing switch of GABA from excitatory to inhibitory varies among brain regions, but in the rodent, it is generally complete after the second week of life (Ben-Ari, 2002; Cherubini *et al.*, 1991). The GABA switch is accomplished in cultured cerebellar granule neurons taken from 6–8-day-old rodents and grown for seven days in appropriate culture conditions (Borodinsky *et al.*, 2003).

In immature cerebellar neurons GABA is capable of increasing intracellular calcium at very low concentrations starting at 0.1 μM with maximal effective concentrations of 100 μM (Borodinsky *et al.*, 2003). The effect of GABA is

blocked by nifedipine, an L-subtype VGCC blocker, and by bicuculline and picrotoxin (a $GABA_A$ receptor blocker and a $GABA_A$ chloride channel blocker, respectively), confirming that the effect is mediated by $GABA_A$ receptors, chloride conductances increases, and activation of VGCC (Borodinsky *et al.*, 2003). During the first days *in vitro* (DIV1-2) depolarizing potassium concentrations increase calcium and the effect is blocked by nifedipine. However, GABA was less effective in triggering calcium increases than 25 mM KCl (Borodinsky *et al.*, 2002, 2003). This is possibly due to the fact that 25 mM KCl activates calcium channels whereas GABA effect is subject to other regulatory factors that may affect $GABA_A$-mediated responses.

The mechanism that explains GABA acting as an excitatory neurotransmitter in early development is an outward chloride gradient, resulting from a higher intracellular concentration of chloride that provides a depolarized chloride equilibrium potential (Rohrbough and Spitzer, 1996). The transporter that mediates the intracellular accumulation of chloride is the Na^{2+}-K^+-2Cl– co-transporter (NKCC1), which is highly expressed in the developing brain (Li *et al.*, 2002; Plotkin *et al.*, 1997). It is generally accepted that the outward chloride gradient in immature neurons is a consequence of an active chloride accumulation via NKCC1 (Ben-Ari, 2002). In more mature neurons, a mechanism that lowers the chloride concentration by extruding chloride, the exporter K^+/Cl^- co-transporter (KCC2), is also expressed. It has been demonstrated that neuronal maturation is concomitant with KCC2 expression (Rivera *et al.*, 1999).

What are the factors that determine the switch from depolarizing to hyperpolarizing GABA? In young cultured hippocampal neurons it has been demonstrated that GABA itself, acting as a depolarizing agent, promotes the switch from depolarizing to hyperpolarizing responses, with a concomitant up-regulation of KCC2 (Ganguly *et al.*, 2001). According to the authors, the effect is specific for GABA, since glutamate blockers failed to prevent it.

In cerebellar granule neurons cultured for 1 to 8 days in 5 mM KCl the exogenous application of 100 μM GABA induced an increase in intracellular calcium concentration with 70% of the cells being responsive (Borodinsky *et al.*, 2003). This differs from cells plated in 25 mM KCl, where the increase in calcium induced by GABA was observed in cells from 1 to 4 DIV but not in cells cultured for 5 days or longer. At DIV5 only 20% of the cells responded to GABA with an increase in calcium. The ionic mechanism that underlies the change in the response to GABA was studied performing perforated patch recordings using amphotericin B in the recording pipette to allow access to the cell without altering its chloride and other intracellular ion concentrations. We recorded from granule neurons as early as 24 h after plating. The resting membrane potential of cells at 1–3 DIV measured in current-clamp perforated patch recordings did not differ in 5 and 25 mM KCl containing cultures. GABA applied to both neurons grown in 5 and 25 mM KCl produced a depolarization. We then compared the

response induced by 100 μM GABA in voltage-clamped cerebellar granule cells at various holding potentials to determine the reversal potential (Erev). When neurons were grown in 25 mM KCl, currents evoked by GABA application to cells' voltage clamped at −45 mV were depolarizing at DIV1 and hyperpolarizing at DIV8. In cells exposed to 25 mM KCl at 1–3 DIV the Erev of GABA currents was 36 ± 1 mV. In contrast, at DIV8-9 the Erev was 47 ± 2 mV. On the other hand, when neurons were grown in 5 mM KCl for DIV6–8, currents evoked by GABA application were still depolarizing and the Erev remained unchanged from DIV1 to 8 (Borodinsky *et al.*, 2003).

These experiments suggest that the switch of GABA responses can be induced in developing cerebellar granule cells by depolarizing potassium concentrations (Borodinsky *et al.*, 2003), a culture condition known to increase NMDA receptor activation (Armanino *et al.*, 2005; Fiszman *et al.*, 2005) and VGCC activation (Borodinsky *et al.*, 2002; Gallo *et al.*, 1987). Furthermore, it was reported that neurotrophic factors can up-regulate KCC2 since overexpression of brain-derived neurotrophic factor (BDNF) in immature hippocampal neurons raises spontaneous activity and synaptogenesis to postnatal levels and up-regulates KCC2 (Aguado *et al.*, 2003). Taken together, these reports suggest that the GABA switch is a multi-factorial phenomenon triggered by different conditions that promote neuronal maturation and differentiation and in the cerebellum, in particular, depolarizing conditions are one of the main regulatory factors.

VIII. Intracellular Pathways Activated Downstream $GABA_A$ Receptors

Intracellular calcium increases activate calmodulin and other related kinases, including CaMKII, which is involved mainly in neuronal plasticity (Cline, 2001). CaMK IV, which plays a crucial role in Ca^{2+}-dependent activation of CREB-dependent transcription (Bradley and Finkbeiner, 2002; Curtis and Finkbeiner, 1999) is also activated and is involved in neurite outgrowth (Redmond et al., *2002*). Other kinases modulated by calcium influx such as p42/44 MAPK (erk1/2), p38-MAPK, and PI3-kinase (Dolmescht *et al.*, 2001; Ghosh and Greenberg, 1995; Rosen and Greenberg, 1996; Rosen *et al.*, 1994) are also involved in neurite outgrowth and plasticity (Borodinsky *et al.*, 2002; Opazo *et al.*, 2003; Vaillant *et al.*, 2002).

In the developing cerebellum, GABA increases the phosphorylated forms of erk 1/2 and CaMKII as effectively as 25 mM KCl (Borodisnky *et al.*, 2003). Therefore, GABA is capable of activating calcium-dependent and neurotrophin-related cascades. In cultured hippocampal neurons GABA can activate BDNF expression and *c-fos* expression through a calcium-dependent mechanism (Berninger *et al.*, 1995) and regulate phenotypic expression of hippocampal

interneurons through an increase in BDNF levels (Marty *et al.*, 1996). The effect on BDNF expression was reported to be accomplished via a MAPK-CREB-dependent mechanism in hypothalamic neurons (Obrietan *et al.*, 2002). Therefore, GABA may act directly by increasing intracellular calcium and/or indirectly by increasing availability of BDNF.

We conclude that GABA, acting as a depolarizing agent, activates intracellular cascades that mediate trophic effects similar to neurotrophins. We next explore the effects of GABA on proliferation, differentiation, and survival of cerebellar neurons.

A. GABA and Proliferation

Depolarization affects cell cycle progression by increasing proliferation in immature central and sympathetic autonomic neuroblasts (Cone, 1980; DiCicco-Bloom and Black, 1989). Amino acid neurotransmitters affect proliferation of immature neuroblasts in different directions. For example, in the embryonic neocortex, exposure to GABA, high potassium, and glutamate decrease proliferation of immature neurons (Lo Turco *et al.*, 1995). In cultured cortical progenitors, $GABA_A$ receptor activation decreases the proliferative effect induced by bFGF (Antonopoulos *et al.*, 1997). Cell cycle progression of cortical progenitors is differently affected by GABA and glutamate in ventricular and subventricular zones of the developing cortex (Haydar *et al.*, 2000). It is interesting to note that even high levels of glutamate receptor agonists are not toxic for the subventricular zone-derived neural stem/progenitor cells *in vitro* and activation of kainate receptors in a perinatal model of ischemia is trophic instead of toxic (Brazel *et al.*, 2005).

We explored the effect of GABA on immature cerebellar granule cells' proliferation. We found that GABA at micromolar concentrations induced an increase in cell proliferation that was mimicked by muscimol (Fiszman *et al.*, 1999), a selective $GABA_A$ agonist. The proliferative effect induced by GABA is mediated by $GABA_A$ receptors and chloride channels' opening since it was completely blocked by bicuculline and picrotoxin. The effect was also blocked by nifedipine and $MgCl_2$ but not by NMDA receptors blockers, suggesting that it is a calcium-dependent effect and voltage-gated calcium channel activation is required. An intracellular calcium increase triggered a downstream activation of the MAPK cascade, and the proliferative effect induced by GABA was completely blocked by PD 98059, a specific blocker of MAPKK (Fiszman *et al.*, 1999), confirming that Ras/MAPK (erk1/2) is involved in the proliferative effect induced by GABA. Moreover, VGCC activation after exposure to high potassium in the same cell preparation increases cell proliferation that is also blocked by PD 98059 (Borodinsky and Fiszman, 1998).

B. GABA and Differentiation

Cells can often generate calcium transients at very early developmental stages and excitability plays a crucial role in the differentiation of developing neurons (Spitzer *et al.*, 2002). Early expressed glutamate receptors regulate neurite outgrowth in hippocampal neurons and embryonic rat motoneurons (Mattson *et al.*, 1988; Metzger *et al.*, 1998; Wilson *et al.*, 2000) and VGCC activation increases neurite outgrowth in central and peripheral neurons (Borodinsky *et al.*, 2002; Redmond *et al.*, 2002; Vaillant *et al.*, 2002). In differentiating neurospheres, intracellular calcium increases correlate positively with the appearance of the GABAergic neurotransmitter phenotype and neurite outgrowth (Ciccolini *et al.*, 2003) and VGCC activation increase calbindin positive hippocampal pyramidal neurons (Boukhaddaou *et al.*, 2000). Intracellular calcium oscillations modulate axon guidance and pathfinding (Gomez and Spitzer, 1999). An increased association of MAP2 with microtubules concomitant to neurite outgrowth was described in cultured sympathetic neurons treated with high potassium (Vaillant *et al.*, 2002). Furthermore, spine outgrowth from dendrites following NMDAR activation is commonly accompanied by motile activity of the nascent spine and rapid changes in actin dynamics (Fischer *et al.*, 1998; Maletic-Savatic *et al.*, 1999).

There are early observations that describe GABA as a morphogen. Several years ago, many morphological changes were described after GABA exposure. For example, *in vivo* application of GABA in the superior cervical ganglion of the rat triggers neurite outgrowth (Wolff *et al.*, 1978). GABA applied *in vitro* induces ultrastructural changes in cultured cerebellar granule cells (Hansen *et al.*, 1984). GABA promotes neurite outgrowth in cultured hippocampal neurons (Barbin *et al.*, 1993) and the effect is mediated by BDNF increases (Marty *et al.*, 1996).

Since GABA activates VGCC and triggers calcium in developing cerebellar granule cells we tested the effect of GABA on the differentiation of these cells. We carried out studies in young post-mitotic cerebellar granule neurons and found that the exogenous application of GABA increases the levels of the phosphorylated forms of CaMKII and erk 1/2, at the same time increasing neurite complexity. The increase in neurite complexity is due to VGCC activation of the L-subtype similar to the effect of high potassium in the same preparation (Borodinsky *et al.*, 2002, 2003). However, while the GABA-induced neuritogenic effect requires the activation of CaMKII and MEK1 (the kinase that activates erk 1/2), high potassium induces an increase in neuronal complexity that was mediated by CaMKII but not by MEK1 activation (Borodinsky *et al.*, 2002, 2003). It is possible that a differential activation of downstream effectors may take place, depending on the magnitude of Ca^{2+} spikes triggered by each stimulus (Dudek and Fields, 2001). Activation of these kinases and neurite outgrowth increases were also described in rat sympathetic neurons exposed to high potassium (Vaillant *et al.*, 2002).

C. GABA and Survival

There are several reasons to believe that GABA may increase neuronal survival. Firstly, depolarizing culture conditions promote and chronic blockade of sodium channels by TTX decreased survival of neurons *in vitro* and *in vivo* (Catsicas *et al.*, 1992; Ruitjer *et al.*, 1991). In the case of the cerebellum, activity plays a crucial role in the survival of neurons rescuing them from death induced by trophic factor deprivation (Gallo *et al.*, 1987). Secondly, GABA increases BDNF expression in developing hippocampal interneurons (Marty *et al.*, 1996) through a calcium-dependent mechanism, (Berninger *et al.*, 1995) suggesting an indirect action of GABA through neurotrophins. We expected that GABA and muscimol would increase cerebellar granule cells' survival in our experiments. However $GABA_A$ agonists failed to increase survival of cultured cerebellar granule neurons (Fiszman *et al.*, 1999). The explanation for this controversy may lie in how and when the GABA switch from depolarizing to hyperpolarizing takes place in our experimental conditions. As mentioned before, this switch is a consequence of maturation processes like neural activity and BDNF-dependent mechanisms that promote differentiation and up regulate KCC2. In the cerebellar granule neurons, activation of VGCC by GABA may induce the GABA switch as we described for cells cultured in high potassium (Borodinsky *et al.*, 2003). It is interesting to note that this high potassium culture condition is effective in promoting survival of cerebellar granule neurons by activating VGCC, (Borodinsky *et al.*, 2002) but, in contrast with previous observations in cortical neurons (Ghosh *et al.*, 1994), the effect is exerted in a BDNF-independent manner (Armanino *et al.*, 2005), suggesting that GABA has no effect on BDNF expression in cerebellar neurons. We postulate that GABA, through VGCC activation, promotes the maturation of the cerebellar granule neurons and induces the GABA switch. Therefore, GABA fails to increase survival because it becomes hyperpolarizing while cerebellar granule cells need a continuous depolarization to keep the majority of cerebellar granule cells alive.

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ROLE OF GABA IN THE MECHANISM OF THE ONSET OF PUBERTY IN NON-HUMAN PRIMATES

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Evidence indicates that GABA is an inhibitory neurotransmitter responsible for restricting luteinizing hormone-releasing hormone (LHRH) release before the onset of puberty. LHRH neurons in the hypothalamus of female rhesus monkeys are already active during the neonatal period, but subsequently enter a dormant state in the juvenile/prepubertal period because of an elevated level of GABA in the stalk-median eminence (S-ME). The developmental reduction in tonic GABA inhibition results in an increase in LHRH release in the S-ME, triggering puberty. The reduction in GABA also appears to allow an increase in glutamate release in the S-ME and this glutamate seems to further contribute to the pubertal increase in LHRH release. These observations conducted in non-human primates, as a model for humans, provide some insights into future studies of the importance of GABAergic mechanisms in the relation between onset of puberty and neurodevelopmental disorders including autism.

I. Introduction

There are several neurological and psychiatric diseases associated with puberty and changes in GABAergic function may, at least in part, be responsible for underlying etiology. First, recent discoveries by several laboratories indicate

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that an abnormality of the GABAergic neuronal system may be a cause of autism or responsible for some symptoms of autism (Cohen *et al.*, 2002; Dhossche, 2002; Hussman, 2001; Menold *et al.*, 2001; Muhle *et al.*, 2004; Nurmi *et al.*, 2003; Prosser *et al.*, 1997). Moreover, in some patients, a worsening of autistic behavior is observed in association with puberty (Gillberg, 1984; Mouridsen *et al.*, 1999). Second, it has been well documented that the onset of schizophrenia occurs between late puberty and early young adulthood (Lewis, 1997; Lewis *et al.*, 2005). A significant decrease in the GABA transporter, GAT-1, immuno-reactivity on axonal terminals of a subset of GABA neurons that innervate pyramidal cells in the frontal cortex is observed in patients with schizophrenia when compared to normal human subjects (Woo *et al.*, 1998). Third, the new onset of epileptic seizures tends to occur early in life and during the adolescent period (Appleton and Gibbs, 1998; Robertson *et al.*, 1990). Precocious puberty is also often associated with epilepsy in children (Elian, 1970; Lennox and Lennox, 1960; Mouridsen *et al.*, 1999; Shenoy and Raja, 2004). Furthermore, treatment with sodium valproic acid, a GABA agonist, delays the timing of puberty in children with seizure disorders (Cook *et al.*, 1992; Lundberg *et al.*, 1986) and in genetically epilepsy-prone mice (Snyder and Badura, 1995). It is possible that the pubertal increase in gonadal steroids may sensitize neurocircuits involved in epileptic seizures, but it is also possible that there is a common mechanism of developmental deficiency (i.e., weakened GABA inhibition in the LHRH neuronal system resulting in precocious puberty and weakened GABAergic inhibition in the brain at the pubertal age resulting in epilepsy) (Olsen and Avoli, 1997). Bourguignon and colleagues treated an 11-month-old child who exhibited severe epileptic seizures and precocious puberty with loreclezole and vigabatrin, GABA agonists. At an earlier stage traditional treatment for epilepsy with phenobarbital was not effective in this patient. However, treatment with loreclezole followed by vigabatrin not only regressed all signs of precocious puberty, but also settled seizure attacks (Bourguignon *et al.*, 1997).

This laboratory has been studying the mechanism of the onset of puberty in the rhesus monkey, as a model for humans. Specifically, results from a series of experiments suggest that the GABAergic neuronal system is, in part, responsible for the timing of puberty in primates (Terasawa, 1995, 2000). Puberty is an important developmental stage during which not only reproductive function is attained (Terasawa and Fernandez, 2001), but also the maturation of the prefrontal cortex, responsible for adolescent behaviors, occurs (Gogtay *et al.*, 2004). Recently, a concept has been proposed that the maturation of the hypothalamus, responsible for puberty, may occur independently from the maturation of the cortices, but there may be common mechanisms governing the maturation of reproductive function and behaviors (Sisk and Foster, 2004). Therefore, a series of observations from this laboratory conducted in non-human primates provide

some insights into better understanding the role of GABA function in the possible relation between onset of puberty and clinical changes in autism and other neuropsychiatric disorders.

II. Developmental Changes in Luteinizing Hormone-Releasing Hormone Release

The decapeptide, luteinizing hormone-releasing hormone (LHRH, also called gonadotropin-releasing hormone or GnRH), is synthesized in the preoptic area and hypothalamus and is released into the pituitary portal circulation in a pulsatile manner at approximately 60 minute intervals in mature primates including humans (Hotchkiss and Knobil, 1994). In juveniles the pulse interval of LHRH release is much longer, 90–120 minutes (Plant, 1994). Acceleration of the pulse frequency accompanied by an increase in the pulse amplitude, hence an increase in total output of LHRH release, triggers the onset of puberty (Watanabe and Terasawa, 1989). It has also been shown that pulsatile administration of LHRH into juvenile monkeys results in precocious puberty (Wildt *et al.*, 1980), and pulsatile administration of LHRH agonist and antagonist analogs has been used for the treatment of precocious and delayed puberty in humans (Crowley *et al.*, 1985).

Although LHRH neurons in the preoptic area and hypothalamus are reasonably mature at birth (Terasawa and Fernandez, 2001) and release the decapeptide in a pulsatile manner shortly after birth, the adult type of secretory pattern with a higher pulse frequency (interval, ∼60 minutes) is not established until puberty. This is probably due to the immaturity of transsynaptic input regulating LHRH neurons before puberty, either by (1) insufficient excitatory neuronal input to LHRH neurons, or (2) by inhibitory neuronal input to LHRH neurons suppressing activity of LHRH neurons. Electrical stimulation of the medial basal hypothalamus in prepubertal monkeys results in LHRH release of the same magnitude observed in pubertal monkeys (Claypool *et al.*, 1990). However, the immaturity of inhibitory input in control of LHRH release is the predominant mechanism over the immaturity of excitatory input before the onset of puberty (i.e., activity of LHRH neurons during the neonatal period is elevated for the first few months after birth in rhesus monkeys and several months after birth in humans, but is then suppressed by an unknown source of inhibition until shortly before puberty) (Plant, 1994). This inhibition is central in origin, independent from suppression by the ovarian steroid hormone estrogen (Chongthammakun *et al.*, 1993; Terasawa *et al.*, 1983). Thus, we investigated inhibitory neurotransmitters for LHRH release before puberty.

At an early stage we examined the role of beta-endorphin, an inhibitory neuropeptide. However, we excluded it as a candidate for prepubertal inhibition of LHRH release: The release of beta-endorphin increased concomitant with the pubertal increase in LHRH release (Terasawa and Fernandez, 2001). Subsequently, we examined the role of GABA, a dominant inhibitory neurotransmitter in the hypothalamus, (Decavel and van den Pol, 1990) and have found that GABA is an important neurotransmitter responsible for the timing of puberty.

III. Developmental Changes in GABA Release

LHRH neurons release the decapeptide into portal circulation located in the median eminence (ME) and pituitary stalk (S), and appear to be controlled by presynaptic input from GABA neurons. Modulation of LHRH neurons by GABA neurons appears to occur at the cell body and dendrites as well as at the neuroterminals. As the first step to assess the role of GABA in puberty, we measured the simultaneous release of LHRH and GABA in the S-ME. The collection of samples from the S-ME located in the base of the hypothalamus in unanesthetized monkeys is a challenging task, but we have been successful in collecting hypothalamic perfusates for the detection of neurochemical substances using a push-pull perfusion method. As described previously (Terasawa, 1994), a double lumen cannula is inserted into the S-ME with aid of x-ray ventriculograms, and artificial CSF is slowly infused to the area, approximately 1 mm^3, through the push cannula while perfusates are continuously collected through the pull cannula using two peristaltic pumps calibrated at identical speeds. Using this method, we are able to measure LHRH, GABA, glutamates, beta-endorphin, neuropeptide Y, prostaglandin E_2, and catecholamines and their metabolites in various physiological conditions (Terasawa, 1994). This method is also useful in examining the effects of neurotransmitter agonists and antagonists on neurotransmitter/neuromodulator release, such as LHRH and/or GABA, by direct application through the push cannula.

Developmental changes in GABA and LHRH levels in the same samples were assessed using this method. Perfusate samples from the S-ME are collected from prepubertal monkeys at 13–21 months of age (before any sign of puberty is apparent), early pubertal monkeys at 22–28 months of age (after some signs of puberty, before menarche), and midpubertal monkeys at 34–46 months of age (after menarche, but before first ovulation). LHRH and GABA levels were measured by radioimmunoassay and HPLC with electrochemical detection, respectively. In prepubertal monkeys LHRH levels are low, whereas GABA levels measured in the same samples are high. LHRH levels significantly increase in

early pubertal and midpubertal monkeys, whereas GABA levels are significantly low in both early and midpubertal monkeys (see Fig. 1; Terasawa *et al.*, 1999). These observations are similar to those reported previously (Mitsushima *et al.*, 1994). Although we were not able to obtain data from monkeys between the neonatal period and 12 months of age because they were not weaned, a scatter plot of GABA levels during the ages of 13 to 46 months (see Fig. 2) indicates that there is a clear developmental GABA decrease. Interestingly, this profile in female rhesus monkeys resembles that described for circulating GABA levels in normal children (Dhossche *et al.*, 2002).

IV. Evidence for GABA as an Inhibitory Neurotransmitter Before Puberty

A question arises as to whether high levels of GABA release in the S-ME before puberty have any physiological significance. To answer this question, two experiments were conducted. First, we examined the effect of the $GABA_A$ receptor antagonist, bicuculline, on LHRH release (Mitsushima *et al.*, 1994). Results suggest that bicuculline stimulates LHRH release in prepubertal monkeys by removing endogenous GABA inhibition, whereas exogenous GABA is not effective in suppressing LHRH release until after the onset of puberty, when endogenous GABAergic tone is reduced. The $GABA_B$ receptor blocker, saclofen, was not effective in prepubertal monkeys. Second, we examined whether lowering GABA levels in the S-ME by chronic infusion of bicuculline triggers puberty (Keen *et al.*, 1999). The average ages of menarche and first ovulation in female rhesus monkeys in our colony are approximately 30 and 45 months, respectively (Terasawa *et al.*, 1983). Pulsatile infusion of bicuculline into the third ventricle of prepubertal monkeys results in precocious menarche, which occurs 6–8 weeks after the initiation of bicuculline infusion, and in precocious first ovulation, which occurs by 30 months, the age of menarche in control females (see Fig. 3; Keen *et al.*, 1999; Richter and Terasawa, 2001). However, since the interval between menarche and first ovulation is not shortened by bicuculline infusion, additional mechanisms, such as the establishment of the stimulatory neuronal system for pulsatile LHRH release, are necessary for the pubertal transition in female primates. The mean ages of menarche and first ovulation in control monkeys receiving saline infusions are not different from the data in colony controls. The results of these two experiments indicate that tonic GABAergic inhibition in the S-ME is, at least in part, responsible for restraining the activity of LHRH neurons before the onset of puberty and reduction in GABAergic inhibition triggers the pubertal increase in LHRH release resulting in the onset of puberty.

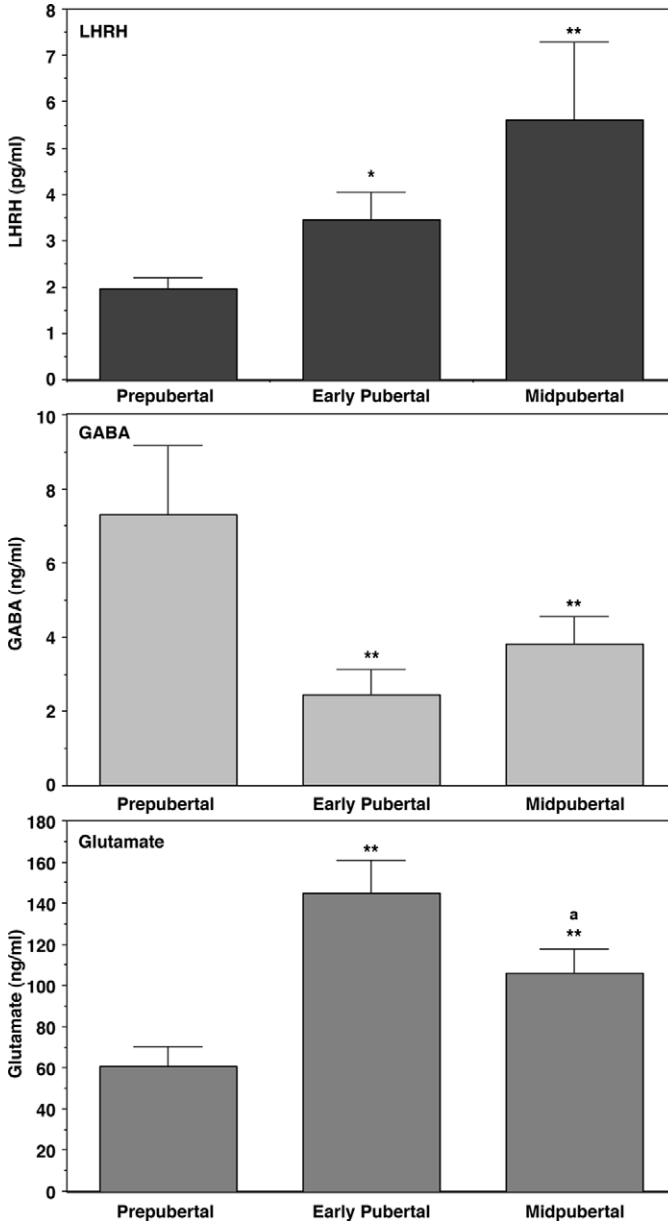

FIG. 1. Developmental changes in luteinizing hormone-releasing hormone (LHRH, top), GABA (middle), and glutamate (bottom) levels in the stalk-median eminence of the hypothalamus in female monkeys. Samples were obtained using the push-pull perfusion method. Note that GABA release in the stalk-median eminence decreases, whereas glutamate release increases, when the pubertal

V. Role of Glutamic Acid Decarboxylase in Puberty

In presynaptic neurons GABA is synthesized from glutamate by decarboxylation in the presence of glutamic acid decarboxylase (GAD), stored in vesicles, and released by exocytosis upon depolarization in the presence of extracellular Ca^{2+} (Rando *et al.*, 1981). There are two different proteins, GAD67 and GAD65, derived from respective genes (Erlander and Tobin, 1991; Rimvall and Martin, 1993). To assess the possible involvement of GADs in puberty, we examined whether interference in GAD67 and GAD65 synthesis in prepubertal monkeys modifies the LHRH release pattern (Mitsushima *et al.*, 1996). Infusion of antisense oligodeoxynucleotides for GAD67 and GAD65 mRNAs into the S-ME of prepubertal monkeys results in a dramatic increase in LHRH release (Kasuya *et al.*, 1999; Mitsushima *et al.*, 1996), presumably due to the reduction in GABA synthesis and subsequent GABA release (Terasawa *et al.*, 1999; Mitsushima *et al.*, 1996). Scrambled oligodeoxynucleotides for GAD67 and GAD65 mRNAs as controls did not induce any significant effect. Observations from this experiment indicate that interference in GAD67 and GAD65 synthesis is effective in reducing tonic GABAergic inhibition, resulting in an increase in LHRH release.

Developmental changes in GABA and GAD in primates are not well studied, and there are conflicting data from the postnatal period. GAD activity in the human neocortex sharply increases at birth and continues to increase until 1 year of age, after which it declines gradually until pubertal age and then slightly increases at adulthood (Diebler *et al.*, 1979; Johnston and Coyle, 1981). Urbanski *et al.* (1998) reported that the distribution pattern and concentration of GAD67 and GAD65 mRNAs in hypothalamic nuclei assessed by *in situ* hybridization in gonadally intact juvenile (~0.6 years of age) male rhesus monkeys were not different from those in adult (~10 years of age) male monkeys, and a report by Plant and his colleagues (El Majdoubi *et al.*, 2000) indicates that GAD mRNA levels in the basal hypothalamus of juvenile castrated male rhesus monkeys did not differ from those in adult castrated male monkeys. Although these data do not appear to support the hypothesis that GAD plays an important role in puberty, more precise developmental studies with the exact regional distribution pattern of GABA neurons in non-human primates, including in females, are needed before conclusions can be drawn.

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increase in LHRH release occurs. The ages of prepubertal, early pubertal, and midpubertal monkeys are 13–20 months (before any sign of puberty), 21–30 months (some signs of puberty, but before menarche), and 34–46 months (after menarche, but before first ovulation), respectively. Ages of menarche and first ovulation in our colony females are ~30 months and ~45 months, respectively. *$p < 0.05$ vs. prepubertal; **$p < 0.01$ vs. prepubertal; [a]$p < 0.05$ vs. early pubertal monkeys. Modified from Terasawa *et al.* (1999) with permission.

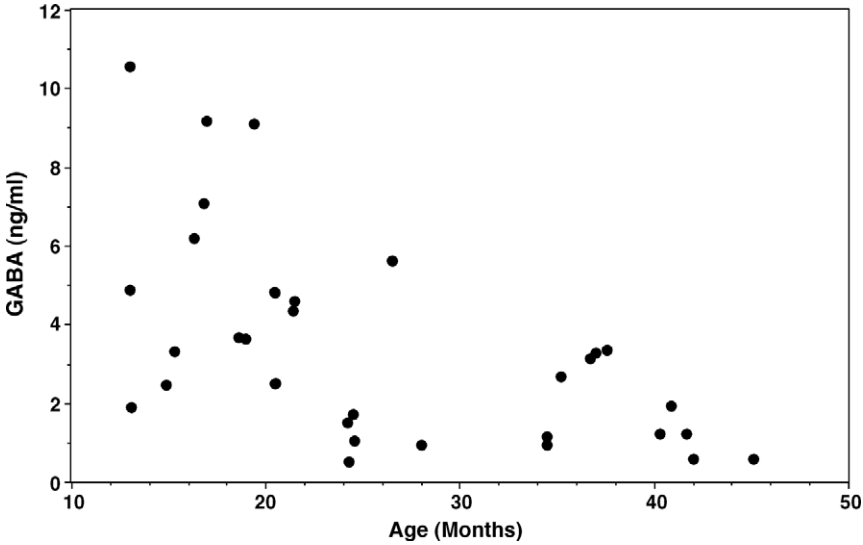

FIG. 2. A scatter plot of GABA release in the stalk-median eminence of female monkeys at ages of 13–46 months. A gradual decline of GABA levels with age is seen.

GAD67 and GAD65 exist in the enzymatically active holo form and inactive apo form, and conversion of holo-GAD67 or holo-GAD65 to and from apo-GAD67 or apo-GAD65 is determined by the presence of the cofactor, pyridoxal-5′-phosphate, which is influenced by physiological states as well as by experimental conditions (Erlander and Tobin, 1991; Kaufman *et al.*, 1991). We do not have any data showing how the ratio of the active and inactive form changes during development.

VI. Other Possible Factors Associated with Developmental Changes in GABAergic Function

A. GABA Transporters

Elevated local concentrations of GABA in the synaptic cleft are actively removed by GABA transporters (GAT), located on presynaptic terminals and surrounding glial cells, where the neurotransmitters are recycled. Four GABA transporters (GAT-1, GAT-2, GAT-3, and BGT-1), classified as the Na^+ and Cl^--coupled transporter family, have been described (Guastella *et al.*, 1990;

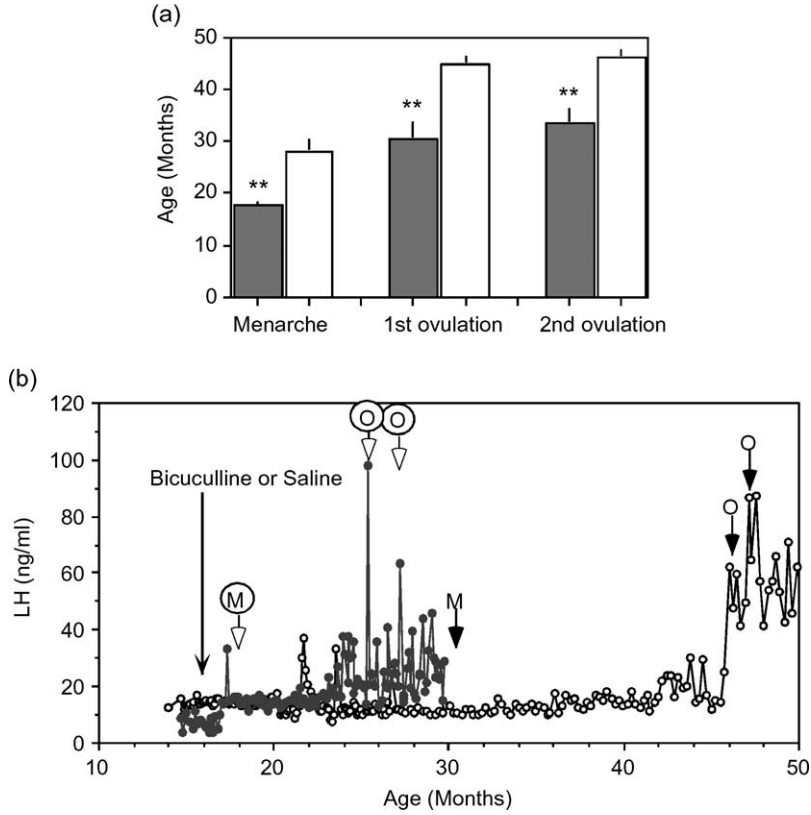

FIG. 3. Reducing GABA neurotransmission with the $GABA_A$ receptor blocker, bicuculline, advances puberty in female rhesus monkeys. (a) The age of menarche and first ovulation in female rhesus monkeys treated by chronic infusion of bicuculline occur at a significantly earlier age than that in controls. Bicuculline treated group, filled bars; saline-treated control group, open bars. **$P < 0.01$. (b) Representative examples showing LH concentration in a bicuculline treated (filled circles) monkey and a saline-treated control (open circles) monkey. The dramatically earlier onset of puberty is shown by menarche (M) and first and second ovulations (O). Modified from Keen *et al.* (1999) and Richter and Terasawa (2001) with permission.

Nelson *et al.*, 1990). GAT-1 is the predominant GABA transporter in the mammalian brain (Borden, 1996) and the GAT-1 transcript contains an estrogen responsive element (Herbison *et al.*, 1995). Thus, it is possible that the reduction of GABA concentration in the S-ME at the onset of puberty might be due to a developmentally regulated increase in GABA transporter activity. At this time there is no information on the role of GAT activity in puberty.

B. $GABA_A$ Receptors and Their Subunit Composition

The developmental pattern of each $GABA_A$ receptor subunit is complex and differs from region to region or neuron to neuron, which ensures the functional heterogeneity of GABA input. Nonetheless, it has been consistently reported that, in general, α_2 subunit expression is very high before birth to shortly after birth, and decreases gradually toward adult levels, whereas α_1 expression is minimal in prenates and then gradually increases after birth until adulthood (Brooks-Kayal and Pritchett, 1993; Fritschy and Mohler, 1995; Fritschy *et al.*, 1994; Hendrickson *et al.*, 1994; Laurie *et al.*, 1992). In fact, it appears that $GABA_A$ receptors containing α_1 subunits gradually replace $GABA_A$ receptors containing the α_2 subunit during postnatal maturation in the rat, monkey, and human brain, and that the increase in the α_1 subunit is an indication of brain maturation (i.e., the onset of synaptic GABA inhibition, whereas β subunits do not generally undergo developmental changes) (Brooks-Kayal and Pritchett, 1993; Hendrickson *et al.*, 1994; Laurie *et al.*, 1992). An example of developmental changes in subunit composition in association with function has been shown: Changes in $GABA_A$ receptor subunit composition in hippocampal neurons preceded the onset of epilepsy by weeks in epileptic rats (Brooks-Kayal *et al.*, 1998).

It is, therefore, possible that developmental changes in the $GABA_A$ subunit composition in neuronal cells may occur prior to the onset of puberty. Analysis of the literature provides support for the hypothesis that GABA disinhibition of LHRH neurons through $GABA_A$ receptors at the onset of puberty in female monkeys (Mitsushima *et al.*, 1994) is due to changes in $GABA_A$ receptor subunit composition. For example, a report using single cell RT-PCR (Sim *et al.*, 2000) suggests that the pattern of LHRH neurons expressing GABA subunits in the POA and medial septum of sexually immature mice at neonatal and juvenile ages is more heterogeneous than that in adults, and it becomes homogeneous when the mice mature. Interestingly, the same authors have reported that sensitivity to GABA in LHRH neurons of juvenile mice is lower than that in adult mice and that the pattern of the dose response curve to GABA in prepubertal LHRH neurons is more heterogeneous than that in adult LHRH neurons (Sim *et al.*, 2000). At this time, we have little knowledge of developmental changes in the $GABA_A$ subunit composition of LHRH neurons in non-human primates.

VII. The Pubertal Reduction in GABAergic Inhibition is Followed by an Increase in Glutamatergic Tone

Glutamate is profoundly involved in pulsatile LHRH release *in vivo* and *in vitro* through NMDA and kainate receptors. NMDA stimulates release of LH and

LHRH in adult rats and monkeys *in vivo* (Bourguignon *et al.*, 1995; Brann and Mahesh, 1997; Olney *et al.*, 1976; Price *et al.*, 1978; van den Pol *et al.*, 1994; Wilson *et al.*, 1982), and glutamate, NMDA, and kainate all stimulate LHRH/LH release in sexually immature monkeys (Gay and Plant, 1987; Medhamurthy *et al.*, 1990), rats (Bourguignon *et al.*, 1989; Brann and Mahesh, 1992; Cicero *et al.*, 1988), sheep (I'Anson *et al.*, 1993), and fetal sheep (Bettendorf *et al.*, 1999) *in vitro* and *in vivo*. Moreover, stimulation of NMDA receptors results in precocious puberty in rats and monkeys (Plant *et al.*, 1989; Urbanski and Ojeda, 1990), whereas administration of the NMDA receptor blockers, MK-801 or 2-amino-5-phosphonovaleric acid (AP-5), delays the timing of puberty in rats (MacDonald and Wilkinson, 1990; Meiji-Roelofs *et al.*, 1991; Urbanski and Ojeda, 1990; Wu *et al.*, 1990). In contrast, the non-NMDA receptor antagonist, 6,7-dinitro-quinoxaline-2,3-dione (DNQX), fails to change the timing of puberty (Brann and Mahesh, 1994). The excitatory action of glutamate on LHRH release may occur not only through NMDA receptors, but also through metabolic receptors. Therefore, the developmental changes in NMDA and kainate receptors are integrated parts of the mechanism of the onset of puberty.

We have measured glutamate release in the S-ME using the push-pull perfusion method (Terasawa *et al.*, 1999). Glutamate levels during the prepubertal period are very low, but increase strikingly during the early pubertal period, and remain high during the midpubertal period, although midpubertal levels decline slightly from early pubertal levels (Fig. 1). However, this observation from monkeys at different ages (cross-sectional study) does not provide the exact timing of glutamate increase during puberty. Fortunately, there is an indication that the pubertal elevation in glutamate release may occur promptly following GABA reduction. The results of the antisense GAD67 infusion experiment in prepubertal monkeys suggest that the reduction in GABA release induced by the antisense GAD67 treatment is followed by an increase in glutamate release for several hours (Terasawa *et al.*, 1999).

Sensitivity to glutamatergic stimulation increases after the onset of puberty. For example, (1) infusion of NMDA into the S-ME at 10 μM-100 μM stimulates LHRH release in pubertal monkeys, whereas only 100 μM NMDA results in LHRH release in prepubertal monkeys (Claypool *et al.*, 2000), and (2) *i.v.* injection of NMDA at 10 mg/kg results in LHRH responses with a longer duration in pubertal monkeys than in prepubertal monkeys (Claypool *et al.*, 2000). Although this increase in the responsiveness of LHRH neurons to NMDA after puberty may, in part, be due to an increase in circulating estrogen, glutamatergic tone is more elevated after the onset of puberty.

VIII. Conclusions

The mechanism of the onset of puberty is complex. As previously discussed, LHRH neurons are reasonably mature at birth and are already active during the neonatal period. However, in primates "central inhibition" suppresses pulsatile LHRH release during the juvenile period. Studies from this laboratory suggest that the GABAergic neuronal system appears to be a substrate for "central inhibition" in primates. When approaching puberty, this GABA inhibition is removed or diminished, and an increase in LHRH release occurs. Subsequently, increases in stimulatory input from glutamatergic neurons as well as new stimulatory input from norepinephrine and NPY neurons (which we did not discuss here, see Terasawa and Fernandez, 2001) and inhibitory input from β-endorphin neurons to the LHRH neuronal system become active to establish the adult type of regulatory mechanism for pulsatile LHRH release. This pubertal increase in LHRH release results in a cascade of events during puberty, such as increases in synthesis and release of gonadotropins, and increases in steroidogenesis and gametogenesis, followed by the appearance of secondary sexual characteristics.

The most important question still remains: What determines the timing to remove "GABA inhibition"? Because many genes in the brain are turned on or turned off to establish a complex series of events occurring during puberty, the timing of spontaneous puberty must be regulated by a master gene or genes, as part of a series of developmental events. We expect that future studies will include a search for genes determining events to remove "GABA inhibition" and genes which ultimately trigger the onset of puberty in primates.

Understanding the mechanism of the onset of puberty in detail is very important, as puberty is associated with onset of or changes in many neurodevelopmental disorders, including autism, schizophrenia, and affective disorder. At this time it is unclear whether the series of events in the hypothalamus with puberty also occur in the higher brain regions where cognitive function is controlled. Future studies to assess whether similar events in the hypothalamus also occur in the higher brain regions that are involved in autism and other disorders will be useful for treatment strategies.

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RETT SYNDROME: A ROSETTA STONE FOR UNDERSTANDING THE MOLECULAR PATHOGENESIS OF AUTISM

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Autism is a common but complex disorder, showing high heritability but elusive genetic etiology. One approach to "deciphering" the etiology of autism is to investigate overlapping pathways with other autism spectrum-disorders with known genetic causes. Rett syndrome, an X-linked disorder caused by mutations in *MECP2*, and Angelman syndrome, an imprinted disorder caused by maternal 15q11-13 deficiency, share many features with autism. Furthermore, maternal 15q11-13 duplications in autism and rare *MECP2* mutations in autism and Angelman syndrome point to genetic overlap between these three disorders. Recent studies on human postmortem brain samples and *Mecp2*-deficient mouse

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models have demonstrated overlapping pathways in the molecular pathogenesis of these disorders. Although originally described as a transcriptional repressor of methylated genes, new roles for MeCP2 in chromatin organization have recently emerged. In addition, the recent demonstration of MeCP2 as a regulator of both *UBE3A* (the Angelman gene) and *GABRB3* (encoding a $GABA_A$ receptor subunit) expression within 15q11-13 has revealed some interesting insights into the genetic and epigenetic pathways common to all three disorders.

The discovery of mutations in *MECP2,* the gene encoding methyl-CpG binding protein 2, as the cause of Rett syndrome (RTT) in 1999 (Amir *et al.*, 1999) has opened up an entirely new line of investigations about the role of DNA methylation and chromatin in the development of the mammalian brain. Furthermore, of the five pervasive developmental disorders (PDD) subtypes, including autistic disorder, RTT, Asperger disorder, disintegrative disorder, and PDD Not Otherwise Specified (PDD-NOS), RTT is the only one with a known genetic cause (Zoghbi, 2003). Could the emerging information from the rapidly progressing field of MeCP2 research therefore be applied to autism in a "Rosetta Stone" approach to understanding the complex genetics of autism? The Rosetta stone was a small but significant stone tablet containing the same message written in Greek and two different Egyptian scripts that was essential to deciphering Egyptian hieroglyphics. Could the discovery of *MECP2* mutations in RTT be as significant a finding for the field of autism?

I. Overlapping Syndromes: Rett Syndrome, Angelman Syndrome, and Autism

Autism is a neurodevelopmental disorder characterized by severe impairments in social interaction and communication. Symptoms generally appear around 1 to 3 years of age and are characterized by stereotyped mannerisms, abnormal preoccupations, lack of pragmatic language and imaginative play, impaired eye gaze, and impaired joint attention (Volkmar and Pauls, 2003). Males with autism outnumber females by approximately 4 to 1, and the frequency in the population has increased dramatically in recent decades to around 1 in 500 children (Services, 2003). Whether the increased reported prevalence is due to increased diagnosis is under debate, but may suggest that environmental factors affect the incidence of autism (Blaxill, 2004). Neonatal or early childhood exposures to mercury, pesticides, or infections have all been suggested as potential environmental triggers of autism (Lawler *et al.*, 2004).

Twin studies, however, have provided compelling evidence for a genetic origin of autism, as the concordance rate is 70–90% for monozygotic twins and 0–10% for dizygotic twins (Zoghbi, 2003). Yet pinpointing the genetic cause of autism remains challenging since many linkage scans show conflicting results for

multiple loci across the human genome (Polleux and Lauder, 2004). Recent estimates have suggested as many as 10 genes may contribute to the risk of developing autism (Muhle *et al.*, 2004). The complexity of the disorder is further complicated by the large range of phenotypes grouped under the single title of autism-spectrum disorders that may be linked more by aberrant biochemical pathways than by specific genetic loci. Research involving autism spectrum disorders with known genetic causes is therefore being used as an approach to understand the molecular pathogenesis of autism and the normal development of the human brain.

Angelman syndrome (AS) is a rare autism-spectrum disorder (approximately 1/20,000) in which children display severe motor problems, mental retardation, ataxia, hypotonia, seizures, absence of language, and inappropriate laughter (Lalande, 1996). In a recent study, 42% of AS patients also were found to meet the diagnostic criteria for autism (Peters *et al.*, 2004). AS is caused by maternal deletions of 15q11-13, deletions or point mutations in *UBE3A*, paternal disomy of chromosome 15, or maternal methylation defects. Prader-Willi syndrome (PWS) is a distinct neurodevelopmental disorder caused by paternal deficiency of 15q11-13 (Lalande, 1996).

Rett syndrome (RTT) is more common than AS, with an incidence of approximately 1 in 10,000 children (Zoghbi, 2003). RTT is characterized by mental retardation, deceleration of head growth, loss of purposeful hand movements, ataxia, loss of language, autistic features, seizures, and respiratory dysfunction. RTT infants appear normal at birth and develop normally until 6 to 18 months when they exhibit a progressive loss of any previous milestones, including any previously acquired language skills. RTT is an X-linked dominant disorder in which approximately 80% of RTT patients have detectable mutations in *MECP2* within Xq28 (Amir *et al.*, 2000; Bienvenu *et al.*, 2000). Although RTT occurs almost exclusively in females, a hemizygous mutation in *MECP2* is compatible with life, as rare cases of males with *MECP2* mutations have been reported (Schanen, 2001). The phenotype of males with *MECP2* mutations is much more severe, with death in infancy or early childhood. The almost exclusive occurrence of RTT in females has been explained by a bias of *de novo MECP2* mutations in the paternal X chromosome (Girard *et al.*, 2001).

Autism, RTT, and AS are all characterized by loss or impairment of language, stereotyped behaviors, and a high frequency of seizures and sleep abnormalities. RTT and AS both cause severe mental retardation, while approximately 70% of autistic patients are also mentally retarded (Polleux and Lauder, 2004). AS is usually apparent at birth, while the onset of both RTT and autism follows a period of apparently normal infant development that is often followed by a period of apparent regression in skills.

Genetic overlap has previously been suggested between AS and autism because the most common cytogenetic abnormality (1–3% of autism cases) is a

maternal duplication of 15q11-13 (Schroer *et al.*, 1998). In addition, the 15q11-13 region has emerged as a candidate autism locus from multiple linkage and association studies (Buxbaum *et al.*, 2002; Kim *et al.*, 2002; McCauley *et al.*, 2004; Nurmi *et al.*, 2001, 2003). In one study, approximately 2% of AS patients had mutations in *MECP2* (Watson *et al.*, 2001) while in another three RTT patients had apparent small deletions within 15q11-13 (Renieri *et al.*, 2003). Several cases of infantile autism with a *MECP2* mutation have been described (Beyer *et al.*, 2002; Lam *et al.*, 2000), however, two large studies have determined that mutations or polymorphisms in the coding region of *MECP2* occur at a low frequency in autistic patients (Beyer *et al.*, 2002; Vourc'h *et al.*, 2001).

The combined phenotypic and genotypic evidence has set the stage for the emerging hypothesis that these three autism spectrum disorders share an overlapping molecular pathogenesis. This review will summarize and discuss the recent evidence supporting this hypothesis by linking MeCP2, expression of genes within 15q11-13, and neuronal nuclear organization in an important molecular pathway that is defective in all three disorders.

II. The 15q11-13 Imprinted Gene Cluster

A. Overview of Imprinting

Genomic imprinting, or preferential gene expression based on parent of origin, was discovered over two decades ago after mice with exclusive maternal or paternal genomes failed to develop (McGrath and Solter, 1984). At least 70 imprinted genes have been identified in mammals and as many as 100–200 imprinted genes are estimated to exist in our genomes (Murphy and Jirtle, 2003). There are several hallmark features of imprinted genes, including CpG islands, repetitive elements near or within the CpG islands, and clustering of maternally and paternally expressed genes (Reik and Walter, 2001). Clustering of imprinted genes likely allows coordinated expression of the differentially expressed genes within the domain and suggests that common control elements regulate imprinting for all genes in the same cluster (Verona *et al.*, 2003). The ~4 Mb imprinted gene cluster on human 15q11-13 is one of the most complex and least understood imprinted loci in the human genome. Imprinted genes within 15q11-13 are implicated in both PWS and AS, clinically distinct disorders that are caused by paternal or maternal deficiencies in 15q11-13, respectively. Due to the significance of this region to human disease much effort has been spent investigating the mechanisms governing gene expression within this complex locus.

B. Phenotypes of Prader-Willi (PWS) and Angelman Syndromes

PWS and AS are both characterized by developmental, neurological, and behavioral abnormalities, although the phenotypes are distinct. PWS patients have hypotonia and growth defects early in life but later in childhood exhibit neurological and behavioral phentotypes such as mild mental retardation, obsessive-compulsive behavior, and obesity due to an insatiable appetite (Lalande, 1996; Nicholls and Knepper, 2001). In contrast, AS patients have severe mental retardation, lack of speech, ataxia, aggressive behavior, and most notably excessive inappropriate laughter. Most commonly these syndromes are caused by a *de novo* deletion of the 15q11-13 chromosomal locus or uniparental disomy of chromosome 15. In AS patients, maternal mutations in the imprinted gene *UBE3A* are also known to cause the disease (Kishino *et al.*, 1997; Rougeulle *et al.*, 1997; Vu and Hoffman, 1997). The protein product of *UBE3A* is a ubiquitin E3A ligase that functions to ubiquitinate proteins thus marking them for degradation via the proteosome (Jiang and Beaudet, 2004). Although *Ube3a* has been shown to affect p53 levels in the brain (Jiang *et al.*, 1998), a paucity of downstream targets have complicated understanding how deficits in *UBE3A* lead to the AS phenotype. No single gene has been identified to independently cause PWS, however the paternally expressed *NDN*, encoding necdin, is considered a strong candidate based on a similar phenotype of the *Ndn*-deficient mouse model (Gerard *et al.*, 1999; Lee *et al.*, 2005; Ren *et al.*, 2003). In a small percentage of patients, PWS or AS is caused by aberrant imprinting and gene silencing due to a reversal of the parental methylation status at the imprinting control region (ICR) (Buiting *et al.*, 2003).

C. 15q11-13 Imprinting Mechanism

The 15q11-13 imprinted cluster contains both maternally and paternally imprinted genes including the paternally expressed *MKRN3, MAGEL2, NDN,* and *SNURF-SNRPN* transcripts and maternally expressed *UBE3A* and *ATP10A* transcripts (see Fig. 1 for more detail). Preferential gene expression within this ~4 Mb region strictly depends on the 4.3 kb imprinting control region (ICR) that overlaps with the *SNRPN* promoter (Sutcliffe *et al.*, 1994). On the maternal chromosome this locus exhibits heavy CpG methylation, while the paternal chromosome is almost completely unmethylated (Glenn *et al.*, 1993). Deletion of the paternal ICR has been observed in PWS patients, demonstrating that this PWS-ICR is required for paternal expression in the 15q11-13 region (Buiting *et al.*, 1995; Sutcliffe *et al.*, 1994). A 900 bp region 35 kb centromeric to the PWS-ICR is termed the AS-ICR because of its maternal deletion in AS patients (Buiting *et al.*, 1995). The AS-ICR establishes the maternal imprint switch during

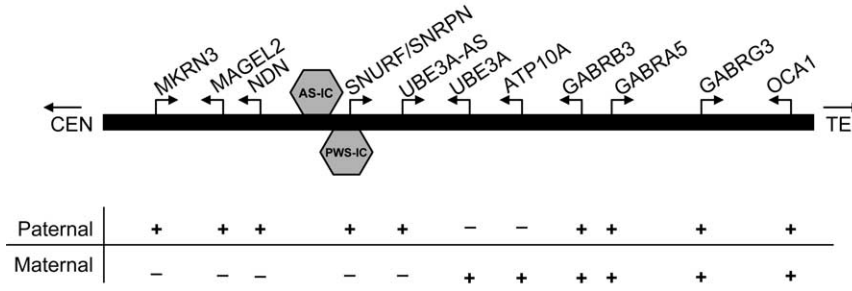

FIG. 1. A schematic map of parental imprinting of transcripts within 15q11-13. The roughly 6 MB region of 15q11-13 that is duplicated in autism patients is shown. Arrows are depicting the direction of each transcript and the expression patterns on the maternal and paternal chromosomes are shown in the lower table. The imprinting control regions for the paternal (PWS-ICR) and maternal (AS-ICR) chromosomes are shown.

early development, however the mechanism involved remains unclear (Buiting *et al.*, 2001; Dittrich *et al.*, 1996). The paternally expressed genes upstream of *SNRPN* exhibit differential methylation, with heavy methylation on the silent maternal chromosome. Unlike the paternally expressed transcripts, *UBE3A* does not exhibit differential methylation but instead is thought to be regulated by a paternally expressed antisense transcript, *UBE3A-AS*, which originates from the *SNRPN* promoter as part of an extensive transcription unit (Runte *et al.*, 2001). The exact termination point of the *UBE3A-AS* transcript is currently unknown and the possibility remains that this transcript also extends through *ATP10A* presumably to regulate imprinted expression of this gene as well. *UBE3A* appears to be imprinted exclusively in postnatal neurons in certain regions of the brain and the antisense transcript is only expressed in regions where imprinting of the paternal *UBE3A* occurs (Yamasaki *et al.*, 2003). Exactly how the ICR regulates imprinted gene expression throughout this entire 4 Mb domain remains unclear.

III. MeCP2: A "Rosetta Stone" for Autism?

A. Biochemical Properties of MeCP2

MECP2 encodes the founding member of a family of methyl CpG binding domain (MBD) proteins (Jorgensen and Bird, 2002). Methylated cytosine in the context of a CpG dinucleotide is associated with heterochromatin and transcriptionally silent genomic DNA, but until the discovery of MBD proteins, how this

epigenetic mark resulted in a silent chromatin state was unclear. MeCP2 was originally isolated from rat brain nuclear extracts based on its ability to bind selectively to methylated CpGs (Lewis *et al.*, 1992). Consistent with a role in transcriptional silencing, MeCP2 was found to colocalize with nuclear heterochromatin (Nan *et al.*, 1996). The MeCP2 MBD domain has since been used to identify four other MBD proteins (MBD1-4) (Hendrich *et al.*, 1999) and has identified *MECP2* among other vertebrates such as frog, chicken, and zebrafish (Coverdale *et al.*, 2004; Jones *et al.*, 2001; Weitzel *et al.*, 1997). In addition to having a conserved MBD, MeCP2 also contains a transcriptional repression domain (TRD) that has been shown to interact with the histone deacetylase complex to direct transcriptional repression of target genes (Jones *et al.*, 1998; Nan *et al.*, 1998). A third domain of a putative binding site for WW-domain proteins (such as splicing factors) has also been described (Buschdorf and Stratling, 2004). *MECP2* mutations in RTT patients are found throughout the gene, but tend to fall into three categories: MBD missense mutations, TRD truncation mutations, and complex rearrangements and deletions in or near the C-terminal WW-binding domain (Zoghbi, 2003).

Although MeCP2 binds specifically to methylated DNA, its action on chromatin usually involves association with additional chromatin modifying enzymes involved in inactivating genes or compacting nucleosomes. MeCP2 interacts with the transcriptional repressor Sin3A and histone deacetylase (HDAC) through the TRD (Jones *et al.*, 1998). In addition, MeCP2 associates with histone methyltransferase activity that directs the silencing histone H3 K9 methylation mark (Fuks *et al.*, 2003). MeCP2 can also interact with the maintenance DNA methyltransferase, Dnmt1, when bound to hemimethylated DNA, providing a mechanism for maintaining inactive chromatin states (Kimura and Shiota, 2003). More recently, MeCP2 was also found to associate with Brahma, a factor normally associated with a chromatin remodeling complex that uses ATP to change nucleosome positions (Harikrishnan *et al.*, 2005). These recently described partners for MeCP2 activity are all consistent with its role in inactive heterochromatin, but are diverging from the originally described role for MeCP2 as a simple transcriptional repressor.

B. Mouse Models

Three different groups have established mouse models of either *Mecp2* deficiency or mutation that recapitulate many of the features of RTT (Chen *et al.*, 2001; Guy *et al.*, 2001; Shahbazian *et al.*, 2002a). Two groups used *Cre-lox* recombination to create *Mecp2*-null mice that show no apparent abnormalities at birth, but developed neurological symptoms around 4–6 weeks of age and died around 10 weeks (Chen *et al.*, 2001; Guy *et al.*, 2001). Conditional deletion of

Mecp2 only in post-mitotic neurons resulted in a similar phenotype (Chen *et al.*, 2001; Guy *et al.*, 2001). Another mouse model, the *Mecp2*$^{308/y}$ truncation mutant mouse, also showed a delayed phenotype with several important features of RTT, including motor impairments, seizures, hyopoactivity, and repetitive stereotyped forepaw clasping (Shahbazian *et al.*, 2002a). The *Mecp2*$^{308/y}$ mouse was also shown to have specific defects in social behavior, making this a potential genetic animal model for autism (Moretti *et al.*, 2005). These mouse models have established that MeCP2 is required for the normal functioning of the mammalian brain and that *Mecp2* mutation is sufficient to cause both RTT and autistic-like phenotypes in mice.

Mecp2 deficiency specifically targeted to post-mitotic neurons is sufficient to cause the neurological phenotypes exhibited in *Mecp2*-null mice (Chen *et al.*, 2001; Guy *et al.*, 2001). Likewise, the phenotypic abnormalities presented in *Mecp2*-null mice have been rescued by expression of transgenic *Mecp2* as a fusion protein with Tau in post-mitotic neurons (Luikenhuis *et al.*, 2004). Interestingly, expression of the same transgenic construct in wild-type mice, resulting in a two-fold increase in expression of *Mecp2*, caused severe motor dysfunction (Luikenhuis *et al.*, 2004). A different transgenic mouse model with 2-fold overexpression of *MECP2* under its own promoter also showed severe neurological abnormalities and death in adult mice (Collins *et al.*, 2004). The intolerance for too much or too little MeCP*2* in mice has exposed the delicate balance of MeCP2 that is required for proper neuronal maturation during postnatal brain development.

C. Developmental and Tissue-Specific Regulation of MeCP2 Expression

Although the earliest investigation of *MECP2* expression by Northern blot analyses suggested ubiquitous expression in all tissues and stages of development (Coy *et al.*, 1999; D'Esposito *et al.*, 1996; Reichwald *et al.*, 2000), multiple recent studies using immunohistochemistry, immunofluorescence, or immunoblot analyses of MeCP2 protein expression have revealed a complex and highly controlled developmental expression pattern in the postnatal brain. In mouse, rat, macaque, and human, MeCP2 expression is heterogeneous in all regions of the brain, with highest expression in mature neurons and lowest expression in glia (Akbarian *et al.*, 2001; Balmer *et al.*, 2003; Braunschweig *et al.*, 2004; Cassel *et al.*, 2004; Cohen *et al.*, 2003; LaSalle *et al.*, 2001; Shahbazian *et al.*, 2002b). Increases in MeCP2 expression follow the developmental maturation of the CNS, with the earliest developing structures in the spinal cord and hindbrain showing highest MeCP2 expression before the cerebral cortex and hippocampus (Braunschweig *et al.*, 2004; Mullaney *et al.*, 2004; Shahbazian *et al.*, 2002b). In rodents, the olfactory bulb also shows high MeCP2 expression (Cassel *et al.*, 2004; Cohen

et al., 2003). Olfactory receptor neurons have been a useful experimental model for determining that increased MeCP2 expression occurs prior to synaptogenesis (Cohen *et al.*, 2003). Neuronal cell lines can also be induced to increase MeCP2 expression with maturational differentiation, consistent with the involvement in pre-synaptogenic events (Mullaney *et al.*, 2004). All of these studies have led to the emerging view that increased MeCP2 expression is a marker of mature neurons that coincides with synaptogenesis in the postnatal brain.

While some of the developmental regulation of MeCP2 is controlled at a transcriptional level (Samaco *et al.*, 2004), the discrepancies between RNA and protein studies for tissue-specific developmental regulation of MeCP2 (Shahbazian *et al.*, 2002b) suggests that multiple post-transcriptional mechanisms play a major role in regulating this dynamically expressed protein. At least four different isoforms exist for *MECP2* transcripts due to alternative splicing and alternative polyadenylation. Alternative splicing of *MECP2*/*Mecp2* exon 2 results in two different transcripts that encode proteins with different N-terminal amino acid sequences due to different translation start sites (Kriaucionis and Bird, 2004; Mnatzakanian *et al.*, 2004). Both MeCP2_e1 (*MECP2B*, *Mecp2*□B&A□(B) and MeCP2e2 (*MECP2A*, *Mecp2*□B&B□(B) contain the three known functional domains and appear to localize similarly to nuclear heterochromatin (Kriaucionis and Bird, 2004). MeCP2_el is more efficiently translated and more highly expressed in brain is more efficiently translated and more highly expressed in brain, but MeCP2_e2 appears sufficient to rescue the RTT phenotype in the *Mecp2*-null mouse when overexpressed (Luikenhuis *et al.*, 2004). Although a few mutations in *MECP2* exon 1 have been described in RTT patients (Mnatzakanian *et al.*, 2004; Ravn *et al.*, 2005), these are a rare cause of RTT (Amir *et al.*, 2005; Evans *et al.*, 2005). The functional significance of the two different N-termini of MeCP2 for the pathogenesis of RTT is still unclear.

The relevance of the two alternatively polyadenylated forms of *MECP2* is also not well understood, although there appears to be both tissue specific and developmental regulation of the process. *MECP2* has an extraordinarily large 3′ untranslated region (3′ UTR) that can add 8.5 kb to the 1.5 kb coding region (Coy *et al.*, 1999; Reichwald *et al.*, 2000). Both 10 kb and 1.9 kb bands are observed by Northern blot, but the long *MECP2* transcript is more prevalent in brain and fetal tissues (Coy *et al.*, 1999). No difference in stability was observed between the two transcripts (Reichwald *et al.*, 2000). In single cell *in situ* analyses of the two transcripts, the ratio of long 3′ UTR transcript to total *MECP2* was reduced with increasing postnatal age, but both transcripts showed higher levels in mature neurons (Samaco *et al.*, 2004).

In addition to variant transcripts of *MECP2*, some evidence for post-translational modifications that may regulate activity of MeCP2 is also emerging. Phosphorylation of MeCP2 was associated with its dissociation from the *BDNF* promoter following activity dependent stimulation of primary neurons (Chen *et al.*, 2003). The combined results of expression, molecular, and biochemical

studies of MeCP2 all suggest that MeCP2 is an intricately regulated protein with multiple levels of control to ensure the correct timing and function in the developing brain. The mouse studies have also demonstrated that the correct dosage of MeCP2 is critical to normal brain development and suggest that multiple *trans* acting factors and pathways could potentially regulate MeCP2 expression.

D. MeCP2 Expression Abnormalities in Autism-Spectrum Neurodevelopmental Disorders

Since *MECP2* is an X-linked gene, RTT females are mosaic for mutant and wild-type expressing cells due to X chromosome inactivation (XCI). In some cases of X-linked dominant disorders skewing towards inactivation of the mutant X chromosome occurs (Hatakeyama *et al.*, 2004). Thus far this does not appear to be the case with *MECP2* mutations in the majority of human females (Zoghbi *et al.*, 1990). Some cases have been reported where a phenotypically normal female is a carrier of mutant *MECP2*, although these cases are relatively rare, as ~90% of RTT mutations are sporadic (Schanen, 2001). In the *Mecp2*-deficient mouse models, however, nonrandom XCI ratios favoring inactivation of the mutant allele were observed in two independent studies (Braunschweig *et al.*, 2004; Young and Zoghbi, 2004). This skewing toward wild-type expression may help to explain why the heterozygous female mice do not develop neurological impairments until adulthood, unlike the onset of RTT in infancy.

In RTT individuals and *Mecp2*$^{-/+}$ female mice, not only is the overall expression of MeCP2 lower, but the wild-type expressing neurons are unable to reach a normal high level of MeCP2 expression (Braunschweig *et al.*, 2004). This indicates that *MECP2* mutation negatively impacts even wild-type expressing neurons in the mosaic RTT brain, potentially by not providing the appropriate local environment for proper neuronal synaptogenesis. Despite having reduced brain size, RTT post-mortem brain tissue is relatively normal. This reduction is due to a decrease in the size of neuronal cell bodies as well as a higher packing density of neurons within several brain regions including the prefrontal, postfrontal, and anterior temporal regions (Subramaniam *et al.*, 1997). In addition, RTT brains have more tightly arranged minicolumn structures, similar to autism brain (Casanova *et al.*, 2002, 2003).

To test the hypothesis that abnormal MeCP2 expression could result from multiple genetic and developmental abnormalities, MeCP2 expression levels were investigated in RTT and other autism-spectrum post-mortem cerebral cortex samples by quantitative immunofluorescence and laser scanning cytometry (Samaco *et al.*, 2004). Although a defect in MeCP2 expression was expected in cases of RTT with known *MECP2* mutations, MeCP2 expression abnormalities were also found in samples from autism, AS, and PWS compared to age-matched

controls. Expression was examined at the RNA level and protein levels and a complex pattern of dysregulation was revealed, suggesting that multiple pathways regulating MeCP2 expression could be aberrant in different autism cases. Although some reports of *MECP2* mutations have been found in a few cases of idiopathic autism (Beyer *et al.*, 2002; Lam *et al.*, 2000), the autism individuals reported lacked coding mutations in *MECP2* (Samaco *et al.*, 2004).

E. MeCP2 Target Genes

Although much effort has been placed in determining the downstream targets for MeCP2 regulation, to date few genes have been found. Expression profiling analysis of RTT and control human brain reported less than 5% of genes significantly altered, with more genes up-regulated than down-regulated (Colantuoni *et al.*, 2001). Microarray analysis of transcriptional differences between *Mecp2*-null and wild-type mouse brain samples also found very subtle effects on gene expression (Tudor *et al.*, 2002). Several careful studies have been successful in identifying genes that are subtly affected by aberrant levels of MeCP2. Two laboratories independently showed that the brain derived neurotropic factor (BDNF) gene is directly regulated by MeCP2 (Chen *et al.*, 2003; Martinowich *et al.*, 2003). A recent study by Horike *et al.* (2005) cloned and sequenced MeCP2 binding sites to screen for MeCP2 target genes. This strategy identified a cluster of imprinted DLX genes that are regulated by MeCP2. Although several other imprinted genes such as *SNRPN* and *H19* have been examined and do not show aberrant expression in the absence of MeCP2 (Balmer *et al.*, 2002; Samaco *et al.*, 2005), *DLX5* showed an apparent loss of imprinting in RTT lymphoblast cultures (Horike *et al.*, 2005).

Other reports (Jiang *et al.*, 2004; Makedonski *et al.*, 2005; Samaco *et al.*, 2005) have speculated that MeCP2 controls gene expression at another imprinted locus in humans and mice, the PWS/AS imprinted cluster at 15q11-13. This locus, illustrated in Fig. 1, contains genes that exhibit parent-of-origin specific gene expression, as discussed in the previous section. Deficiency in MeCP2 results in a decrease in gene expression of two genes within this locus, *Ube3a* and *Gabrb3*, although it does not affect expression of a paternally expressed transcript, *Snrpn* (Samaco *et al.*, 2005). MeCP2 has been shown to physically bind to the imprinting control region (ICR) on the methylated maternal allele of the *Snrpn* promoter (Makedonski *et al.*, 2005; Samaco *et al.*, 2005; Thatcher *et al.*, 2005), however MeCP2 deficiency does not result in maternal *Snrpn* transcription (Balmer *et al.*, 2002; Samaco *et al.*, 2005). Curiously, MeCP2 was not found to be associated with the promoter of either *Ube3a* or *Gabrb3* although expression of these genes is significantly reduced in the absence of MeCP2 (Samaco *et al.*, 2005). Although Makedonski (2005) reported aberrant maternal expression of the *Ube3a-as*

transcript in *Mecp2*-deficient mice, the loss of imprinting was not observed in a different mouse strain cross and no difference in the expression level of *Ube3a-as* was observed (Samaco *et al.*, 2005). Presumably other factors in addition to the *Ube3a-as* can control the level of expression of *Ube3a*.

Surprisingly, reduced expression in both RTT and *Mecp2*-deficient mouse brain was also observed for *GABRB3/Gabrb3*, located ~1 MB distal to *UBE3A/Ube3a* (Samaco *et al.*, 2005). Although no direct binding of MeCP2 to the *GABRB3 CpG* island has yet been reported, regulation of this gene by MeCP2 may resemble that of *Dlx5* (Horike *et al.*, 2005) or *Igf2/H19* loci (Murrell *et al.*, 2004), with long-range effects due to chromatin loop structure.

IV. GABRB3 Expression in Normal Development and Autism

A. The $GABA_A$ Receptor Gene Family

GABRB3 and two other $GABA_A$ receptor subunit genes (*GABRA5* and *GABRG3*) present on 15q11-13 are attractive candidate genes for autism due to evidence for positive linkage, common occurrence of 15q11-13 cytological abnormalities, functional relevance to autistic features, and recent molecular evidence of high frequency expression defects in autism brain. GABRB3 is one of 19 different mammalian subunits that compose the pentameric $GABA_A$ receptor (Barnard *et al.*, 1998). This transmembrane receptor, upon binding of $GABA_A$ forms a chlorine channel that allows rapid membrane depolarization and resulting inhibitory synaptic neurotransmission in the brain. Although there are seven different classes of receptor subunits, the α, β, and γ subunits comprise the majority of $GABA_A$ receptors (Barnard *et al.*, 1998). There are six different α subunits, four β subunits, and three γ subunits which are used in different stoichiometries to form the majority of $GABA_A$ receptors (Pirker *et al.*, 2000). The $GABA_A$ receptor genes exhibit an interesting distribution in the human genome in that all are present in clusters consisting of at least one of each of the three main subunits (McLean *et al.*, 1995). $GABA_A$ receptor clusters are present on chromosomes 4p11-13, 5q34-q35, 6q14-q21, 15q11-13, and Xq28. These clusters are hypothesized to have originated from gene duplication events from an ancestral $GABA_A$ receptor cluster (McLean *et al.*, 1995).

Despite the large variety of subunits not all $GABA_A$ receptor subunits are expressed equally. Extensive studies of the mRNA and protein distributions in rat brain have shown that $\alpha 1$ and $\alpha 2$ as well as all three β subunits are widely distributed throughout the brain (Laurie *et al.*, 1992; Pirker *et al.*, 2000). These data indicate that these constituents are likely present in almost all varieties of $GABA_A$ receptors, while other subunits including the $\gamma 3$ subunit are restricted to

very few brain areas (Pirker *et al.*, 2000). The expression patterns of the $GABA_A$ receptor subunits are consistent with the phenotypes of mouse knockouts of the syntenic 15q11-13 $GABA_A$ receptor cluster on murine 7qB4. A deletion that included the mouse *p* locus (pink eye dilution, the mouse homologue to the *OCA2* gene in human 15q11-13) through the $GABA_A$ receptor cluster was mostly lethal at birth, with rare severely neurologically affected survivors (Nicholls *et al.*, 1993). Mice with deletions encompassing only the *Gabrg3* or both *Gabrg3* and *Gabra5* chromosomal regions were phenotypically normal, indicating that the lethality seen in the larger deletions was due to absence of *Gabrb3* (Culiat *et al.*, 1994). The importance of *Gabrb3* was further illustrated in mice with a disruption of the promoter and exons 1–3 of *Gabrb3* that exhibited a high frequency of neonatal lethality and reduced lifespan (Homanics *et al.*, 1997). In addition, the phenotypic manifestations of *Gabrb3* disruption included cleft palate, presumably contributing to the early fatalities, and neurological abnormalities including hyperactivity, hypersensitive behavior, and seizures (DeLorey and Olsen, 1999; DeLorey *et al.*, 1998). The vast difference in the severe phenotypic manifestation of removal of the *Gabrb3* subunit and the normal phenotype in mice with deletions of the *Gabra5* and *Gabrg3* subunits indicates that *Gabrb3* is an important constituent of $GABA_A$ receptors in the brain.

B. Imprinting Status of the 15q11-13 $GABA_A$ Receptor Cluster

Early mouse studies involving deletions of the $GABA_A$ receptor cluster on mouse chromosome 7 (syntenic to human 15q11-13) demonstrated that expression of these genes is biallelic (Nicholls *et al.*, 1993). In addition, analysis of a single nucleotide polymorphism (SNP) in interspecies hybrid and wild-type mice showed biallelic expression of *Gabrb3* in mouse brain (Nicholls *et al.*, 1993; Yamasaki *et al.*, 2003). However, several studies have suggested that parental origin affects *GABRB3* expression. Since polymorphisms within coding genes are rare in human $GABA_A$ receptor genes, determining parent-specific expression can be difficult. A study by Meguro *et al.* (1997) utilized microcell-mediated chromosome transfer to produce hybrid mice with a single human chromosome 15. Cell lines containing maternal or paternal human chromosome 15 were analyzed for expression of known imprinted genes and found to maintain proper paternal-only expression of *SNRPN* and *IPW*. Expression of the three $GABA_A$ receptor genes in this system only occurred when the human chromosome was of paternal origin (Meguro *et al.*, 1997). Although this system is artificial, two recent reports from actual human samples corroborate preferential paternal expression of the 15q11-13 $GABA_A$ receptor genes (Bittel *et al.*, 2003, 2005). These two studies conducted 15q11-13 gene expression profiles of lymphoblasts from individuals with AS and PWS, arising from 15q11-13 deletions or uniparental

disomy. Comparing cell lines with AS and PWS, *GABRB3* and *GABRA5* showed paternal expression bias (Bittel *et al.*, 2003, 2005). These results pose interesting considerations for the 15q11-13 $GABA_A$ receptor cluster containing candidate genes for autism. Most linkage studies presume equal parental inheritence, however if the gene is only expressed from one allele then significant findings may be masked. Recently, an association study was conducted for $GABA_A$ receptor genes and alcohol dependence (Song *et al.*, 2003). In this study, upon considering a model of paternal expression of *GABRB3* and *GABRA5*, significant association was found, while no significant findings were reported for maternal transmission. Such considerations may be important in analyzing *GABRB3* contribution to autism as well as in searches for candidate genes in autism pedigrees.

C. Genetic Linkage and Association Studies of *GABRB3* and 15q11-13 in Autism

Since autism is a complex trait with potential oligogenic or polygenic inheritance, genome-wide scans for susceptibility alleles have yielded inconsistent results. To date multiple genome-wide scans have been completed and the results regarding 15q11-13 are mixed (Wassink and Piven, 2000). Early studies of linkage disequilibrium supported a role for chromosome 15 in autism (Cook *et al.*, 1998) although this locus has not been consistently identified in all scans (Martin *et al.*, 2000). Although genome-wide approaches with widely distributed markers have failed to find highly significant linkage evidence for 15q11-13, more recent association studies with dense mapping over smaller regions, as well as use of phenotypic subtypes have increased the evidence for autism susceptibility genes in 15q11-13 (McCauley *et al.*, 2004; Nurmi *et al.*, 2003; Shao *et al.*, 2003). One study used dense SNP coverage across the maternally expressed regions of 15q11-13 and found significant evidence for transfer of susceptibility in two SNPs around *ATP10A* upstream of *GABRB3* (Fig. 1) (Nurmi *et al.*, 2003). A later investigation specifically analyzed the $GABA_A$ receptor cluster on 15q11-13 with a dense coverage of markers and found marginally significant evidence for association with *GABRB3* and *GABRA5* (McCauley *et al.*, 2004). Perhaps the most significant evidence for linkage within 15q11-13 was gathered by using a subtype of autism families with the characteristic of insistence on sameness (IS) (Shao *et al.*, 2003). The LOD score for the *GABRB3* locus was increased from 1.45 (insignificant) to a highly significant score of 4.71 by narrowing the analysis down to only families sharing high scores for the IS character. Since autism is a complex disorder with multiple etiologies, the use of phenotypic subtypes, or endophenotyping, may be necessary to remove much of the noise generated from genome scans for linkage.

D. Cytogenetic Abnormalities of 15q11-13 in Autism

Despite the variable support for autism candidate genes by linkage and association studies, the cytogenetic support for 15q11-13 genes' involvement in autism is compelling. Duplications of maternal 15q11-13 are the most common cytogenetic abnormalities reported in autism, occurring in 1–3% of autistic individuals (Cook *et al.*, 1997). Duplications of 15q11-13 are the reciprocal meiotic products of a maternal deletion that results in the related neurodevelopmental disorder Angelman syndrome (AS). AS individuals often exhibit comorbid autism (Peters *et al.*, 2004), which further indicates that abnormalities in genes in 15q11-13 are associated with autism. Deletions of 15q11-13 are the most common cause of AS and result in a more severe phenotypic manifestation of the disease than mutations in *UBE3A*. The phenotypic disparity in AS patients with large deletions and *UBE3A* mutations indicates that the $GABA_A$ receptor genes contribute to the phenotype of this disorder (Moncla *et al.*, 1999). Since autism 15q11-13 abnormalities usually arise from maternal duplications it is unclear how gene expression is modified. One report has indicated that expression of *UBE3A* in a lymphoblast cell line with maternal 15q11-13 duplication is increased (Herzing *et al.*, 2002), however the expression of other genes within the duplicated region, including the $GABA_A$ receptor genes, has not yet been examined. Thus, although 15q11-13 abnormalities are clearly associated with autism, the consequences of the duplication and the effect on gene expression still remain unclear.

E. Abnormal Expression of GABRB3 in Autism Brain

Based on evidence for the involvement of 15q11-13 genes in autism disorder, molecular studies have been conducted to examine expression of genes within this region (Jiang *et al.*, 2004; Samaco *et al.*, 2005). Jiang *et al.* (2004) analyzed UBE3A protein levels in a panel of autism post-mortem cerebellum and cerebral cortex samples, and found reduced levels of UBE3A in some but not all autism samples compared to controls. This result was also confirmed by Samaco *et al.* (2005) using an independent method of quantitative immunofluorescence and laser scanning cytometry. Additionally, expression of GABRB3 was analyzed and significant reduction was found in more than 50% of autism brain samples (Samaco *et al.*, 2005). Other related neurodevelopmental disorder samples including AS and RTT were also analyzed and found to have lower levels of GABRB3 expression (Samaco *et al.*, 2005). Although this result is expected for AS patients with a deletion of maternal 15q11-13, reduced GABRB3 levels in RTT, a genetically distinct condition, is surprising. In addition to sharing a defect in GABRB3 expression, several of the same post-mortem autism brain samples

have been shown to exhibit defects in MeCP2 expression (Samaco *et al.*, 2004). Direct evidence for a causal effect of MeCP2 on 15q11-13 genes was obtained by examining gene expression of *Ube3a* and *Gabrb3* in *Mecp2* null mice. Both *Ube3a* and *Gabrb3* were found to be reduced in MeCP2 deficient mice, although the levels of reduction were not as significant as AS and autism human samples (Samaco *et al.*, 2005). Furthermore, an independent study confirmed that UBE3A expression is reduced in RTT brain samples and *Mecp2* deficient mice (Makedonski *et al.*, 2005).

Despite affecting the level of expression of *UBE3A* and *GABRB3*, MeCP2 does not affect the expression level of the paternally expressed *SNRPN* (Balmer *et al.*, 2002). Even more surprising is the result that MeCP2 binds to the methylated *SNRPN* promoter on the maternal allele of the PWS-ICR (Makedonski *et al.*, 2005; Samaco *et al.*, 2005; Thatcher *et al.*, 2005), but not to the promoters of *Ube3a* or *Gabrb3* (Samaco *et al.*, 2005). Although a model involving MeCP2 regulation of *UBE3A* through loss of imprinting of the maternal *UBE3A-AS* transcript has been proposed (Makedonski *et al.*, 2005), this model fails to explain how MeCP2 regulates other genes, such as *GABRB3*, within the region. Thatcher *et al.* (2005) have reported that homologous pairing occurs at chromosome 15 at the *GABRB3* locus during normal development in human cerebral cortex. Interestingly, this pairing is absent in autism, RTT, and AS samples. Perhaps a new model that incorporates emerging hypotheses about higher order chromatin and nuclear organization is justified in light of these apparently paradoxical new findings. Thus, elucidation of the mechanism used by MeCP2 to regulate gene expression of 15q11-13 may help explain a pathway important in the complex etiology of autism.

V. The Dynamics of Chromatin and Nuclear Organization within the Maturing Neuron

A. The Dynamic Interphase Neuronal Nucleus and Global Transcriptional Regulation

Chromosomal organization is most commonly considered in a metaphase state, and chromosomal movements are best studied in the context of cells undergoing mitosis or meiosis. The organization of chromosomes in interphase is often disregarded. Recent studies using tools to determine nuclear organization, however, have revealed the highly organized and dynamic chromosome architecture within the interphase nucleus. Non-cycling differentiated cells can vary in shape, nuclear volume, size and number of nucleoli, and nuclear protein distribution even more than their replicating counterparts. In addition, the organization and distribution of condensed heterochromatin is similarly

maintained within a given cell lineage but can differ significantly between cell types (Leitch, 2000).

Nuclei can undergo highly dynamic changes in their DNA and chromatin organization even after initial differentiation. This is particularly evident in the mammalian neuronal nucleus which undergoes a highly ordered pattern of chromatin re-organization during neuronal maturation (Manuelidis, 1990; Martou and De Boni, 2000). In immature neurons the nucleus is small and heterochromatic and contains multiple nucleoli. As the neuron matures its nucleus increases greatly in size and a large single nucleolus begins to form (Manuelidis, 1990). The nucleus becomes highly euchromatic with the heterochromatin centralized around the nucleolus and in a complex web-like network throughout the nucleus. In murine neurons the heterochromatin becomes concentrated into two large heterochromatic foci located on either side of the large single nucleolus. These changes in neuronal nuclear organization with maturational differentiation coincide with an increase in levels of transcription and the need for a large nucleolus to transcribe sufficient ribosomal RNA to efficiently translate the new transcripts.

Dynamic chromosome movements have been previously implicated in neuronal maturation and neurodevelopmental disorders. Global nuclear changes in Purkinje neurons located in the cerebellum have been experimentally linked to increased dendritic differentiation and synaptic maturation (Martou and De Boni, 2000). In addition, changes in X chromosome positioning within the human neuronal nucleus have been shown to coincide with epilepsy and changes in cellular activities (Borden and Manuelidis, 1988). Interestingly, addition of gamma-aminobutyric acid (GABA) to neurons has been shown to significantly alter the arrangement of heterochromatin at chromosome centromeres, suggesting a new transcriptional state (Holowacz and De Boni, 1991).

B. Multiple Layers of Transcriptional Regulation

Gene regulation is a highly ordered process involving multiple layers of regulation, from DNA methylation, histone modifications, and nucleosome assembly on linear DNA to spatial positioning of chromosomes in nuclear three dimensional space. Linear gene positioning and clustering with respect to repetitive DNA and other genes can often dictate a gene's transcriptional activity. CpG methylation often regulates vertebrate gene transcription (Bird, 1984) and serves an important role in regulation of imprinted genes (Li *et al.*, 1993). Nucleosome compaction of linear DNA can be modified by methylation, acetylation, phosphorylation, and ubiquitination of histone tails (Jenuwein and Allis, 2001). Modifications of these histones create a "histone code" that alters nucleosome structure leading to changes in DNA accessibility to transcriptional machinery,

transcription factors, and other nuclear proteins (Jenuwein and Allis, 2001). Nucleosomes serve to compact the expansive amounts of DNA found in each cell, acting at the primary level of chromatin structure. A secondary conformation is made of chromatin loops anchored to a nuclear matrix scaffold. Chromatin loops have now been detected by new molecular techniques in mouse and Drosophila, and range in size from 15–400kb (Byrd and Corces, 2003; Chambeyron and Bickmore, 2004).

All of these local modifications to single genes or coordinately regulated gene clusters are part of a larger global organization of the nucleus that affects genome-wide expression. Chromosome organization appears to be an ordered arrangement in the interphase nucleus as seen by fluorescent *in situ* hybridization (FISH) of whole chromosomes (Cremer *et al.*, 1993). Each chromosome appears to maintain fairly distinct chromosome territories with only partial overlap between chromosomes (Kosak and Groudine, 2004). Reviews have speculated that transcriptional regulation is one determinant of a highly organized interphase nucleus (Kosak and Groudine, 2004; Leitch, 2000). Precise nuclear positioning of a chromosomal domain may facilitate the expression of the genes within that region by making it more or less accessible to transcription factors and enable *cis* or *trans* interactions with enhancers. A gene's activity can often be correlated to its nuclear position and distance from constitutive heterochromatin (Brown *et al.*, 1997, 1999, 2001; Francastel *et al.*, 1999; Wang *et al.*, 2004). Initial studies showed that active genes were more susceptible to DNAse I degradation than inactive genes, providing evidence that active genes are in a more open chromatin structure (Weintraub and Groudine, 1976). As a recent example, the murine *Hoxb* gene has been shown by FISH to move out of its chromosome territory upon transcriptional activation and back inside its territory when inactive (Chambeyron and Bickmore, 2004). Active genes from different chromosomes have also been shown to colocalize in specific areas of the nucleus, indicating nuclear regions of increased transcriptional activity and movement outside of chromosome territories (Osborne *et al.*, 2004).

Originally, transcription machinery was thought to be assembled at each active gene throughout the genome regardless of the gene's location. However, recent data suggest that transcription may be localized to discrete areas of the nucleus known as transcription factories where transcribed genes are localized (Jackson *et al.*, 1993, 1998). Evidence for transcription factories includes staining patterns for RNA polymerase II, the primary polymerase of gene transcription, that show punctuate foci staining in multiple cell types pinpointing discrete areas of transcriptional activity (Iborra *et al.*, 1996). The number of RNA polymerase II foci within a single nucleus are too few to account for only one gene being transcribed at each focus (Grande *et al.*, 1997; Zaidi *et al.*, 2002). Active genes from multiple chromosomes which have been shown to localize together by FISH have also been shown to colocalize with a single focus of RNA polymerase II (Osborne *et al.*, 2004).

Transcription factories are thought to interact with clusters of active genes looping out of their chromosome territories, resulting in "active chromatin hubs." One of the best characterized active chromatin hubs, the human β-globin locus, has been found to have a dynamic loop structure which brings active genes in the region into direct spatial orientation with the locus control region (LCR) (de Laat and Grosveld, 2003). Hypersensitive sites are located close to the LCR while the inactive genes are looped out away from the LCR and hypersensitive sites. Deletions of the LCR and specific hypersensitive sites showed that these regions are critical for loop formation (Patrinos *et al.*, 2004). The region is stabilized by *cis*-regulatory elements and high levels of histone acetylation protecting against heterochromatic silencing. Interestingly, in brain, where none of the genes in the β-globin locus are active, the region forms a linear structure in the interphase nucleus (Tolhuis *et al.*, 2002).

A similar active chromatin hub and looping structure may facilitate imprinted gene expression since most imprinted genes occur in clusters and often appear to be regulated by an ICR or differentially methylated region (DMR) that controls gene expression over long distances. These features of long-range control and association with hypersensitive sites make ICRs similar to the LCR of the β-globin locus. PWS and BWS ICRs, for example, regulate gene imprints bi-directionally over several megabases (Soejima and Wagstaff, 2005). Chromatin loop structure is determined by the methylation status, histone modifications, or proteins interacting with the ICR or DMR and can dictate the expression pattern of each gene within the region. Evidence to support this model has been shown at the *Igf2/H19* imprinted locus in mouse. The DMRs in this region interact to produce distinct maternal and paternal chromatin loops that regulate the differential gene expression of each homolog (Murrell *et al.*, 2004).

The combined concepts of transcription factories and active chromatin hubs create a dynamic model by which active gene regions loop out of heterochromatin to transcription factories to be brought into proximity with not only RNA polymerase II, but possibly many other nuclear proteins and enzymes which can facilitate interactions that regulate transcription. This exciting concept of dynamic chromosomal changes in the context of a global nuclear architecture opens a new realm of possibilities to investigate gene regulation during cellular differentiation and maturation.

C. A Role for MeCP2 in Transcriptional Regulation and Chromatin Organization

MeCP2, classically defined as a methylated DNA binding protein that represses gene expression, now appears to have multiple roles at different levels of gene regulation. In the simplest transfected constructs, MeCP2 has been shown

in vitro to repress genes with methylated CpG promoters through its transcription repression domain (TRD) (Nan *et al.*, 1997). MeCP2 has also been shown to interact with histone deacetylase 1 (HDAC) (Jones *et al.*, 1998; Nan *et al.*, 1998) and histone methyltransferase (Fuks *et al.*, 2003) and is therefore thought to be involved in increased histone methylation which generally leads to further DNA compaction and silencing. In addition, MeCP2 has the ability to bind non-methylated DNA when present in high concentrations and to compact naked DNA independently of other factors (Georgel *et al.*, 2003).

Recently Bowen *et al.* (2004) proposed both enzymatic and structural models for MeCP2's involvement in DNA silencing and compaction. The enzymatic model predicts that MeCP2 binds to methylated CpGs and recruits histone modifying enzymes to repress transcription. The structural model suggests a role for MeCP2 in chromatin compaction independent of histone modifiers. Bowen *et al.* (2004) suggest a mixture of the two models, particularly in brain, where higher levels of MeCP2 may even cause MeCP2 to interact with itself or with nucleosomes to create conformational chromatin changes. The staining pattern of MeCP2 in neurons specifically to areas of heterochromatin in the nucleus supports MeCP2 as a factor involved in both gene silencing and heterochromatin condensation (see Fig. 2).

MeCP2 expression is particularly high in mature neurons which have increased transcription and euchromatin (Balmer *et al.*, 2003). This relationship may be a function of the high level of chromatin organization in the mature neuron that requires careful packaging and silencing of specific regions of chromatin while allowing high levels of transcription from others. MeCP2 may also serve to silence genes that inhibit terminal differentiation of neurons. Additionally, MeCP2 may contribute to global chromatin organization by mediating the formation of chromatin loops, and may be of particular importance in the transcriptional regulation of complex clusters of imprinted genes, many of which are expressed in brain. Evidence for this has recently been shown in mouse brain where MeCP2 creates a 11 kb silent-chromatin loop at the *DLX* imprinted region (Horike *et al.*, 2005). The silent loop is absent in *Mecp2* deficient male mice where expression of *DLX5* is two times greater than in wild-type mice. The MeCP2 mediated silent chromatin loop is associated with methylated histone H3 K9, while the active loop, found in the *Mecp2* deficient mice is associated with acetylation of histone H3 K9 and K14.

While no loop structures have yet been defined for the imprinted 15q11-13 locus, three recent papers have demonstrated that MeCP2 binds to the maternal methylated allele of the *SNRPN/Snrpn* promoter in human and mouse brain and human neuroblastoma cells (Makedonski *et al.*, 2005; Samaco *et al.*, 2005; Thatcher *et al.*, 2005). This region is also associated with dimethylation of histone H3 K9 on the maternal allele, and is hyper-acetylated on the paternal allele (Soejima and Wagstaff, 2005). While MeCP2 was not found to be associated with

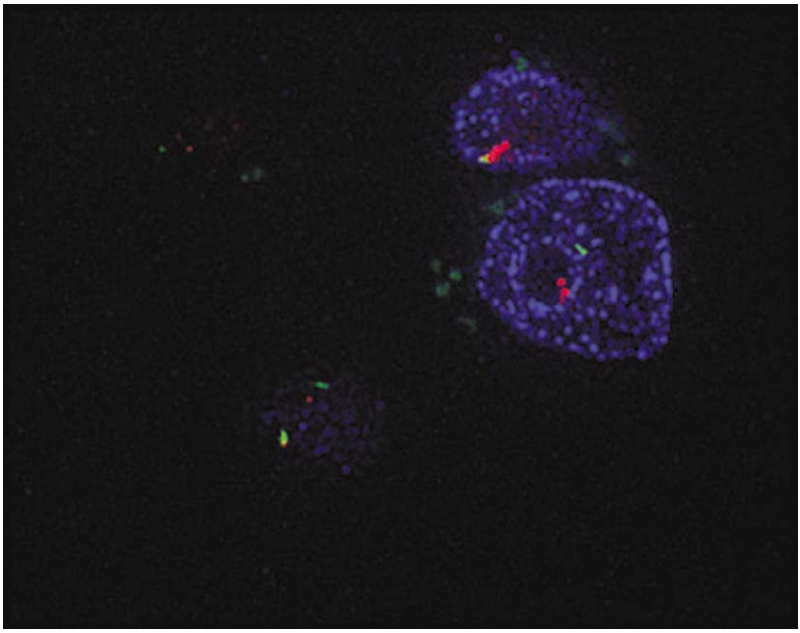

FIG. 2. Homologous pairing of 15q11-13 loci within MeCP2 high expressing neurons in human brain. A normal human cerebral cortical section stained for MeCP2 (blue), chromosome 15 centromere (green), and 15q11-13 (*SNRPN*, red). Two nuclei with high levels of MeCP2 expression (top right) show homologous association of 15q11-13 domains around the nucleolus. In contrast, a neuronal nucleus with low MeCP2 expression (bottom left) shows no homologous pairing.

either the promoter for *Ube3a* or *Gabrb3* (Samaco *et al.*, 2005), MeCP2 may bind at other promoters, perhaps at *NDN* or *MKRN3* which are differentially methylated (Soejima and Wagstaff, 2005). Alternatively, MeCP2 may bind to intergenic or intronic regions throughout 15q11-13 to produce maternal or paternal specific chromatin loop structures, thus regulating transcription of the entire region. Interactions of MeCP2 binding sites along 15q11-13 may even facilitate the interaction of separate gene clusters on different chromosomes.

E. MeCP2 and Homologous Pairing of 15q11-13

Chromatin movement in the neuronal interphase nucleus may facilitate the ongoing maturation and growth of mammalian neurons by orienting genes for transcription that promote increased dendritic branching and synaptogenesis. This dynamic organization may also regulate imprinted genes, many of which are important for proper brain development.

Specific homologous pairing of chromosomes has been previously described in neuronal nuclei and Sertoli cells including association of 9q12 and 1q12 with the nucleolus (Manuelidis, 1990; Manuelidis and Borden, 1988) and somatic pairing of homologous chromosomes 1 and 17 (Arnoldus *et al.*, 1989, 1991). The phenomenon of homologous pairing in interphase nuclei is generally limited to non-cycling differentiated cells (Leitch, 2000), however, transient somatic pairing of 15q11-13 and the centromere of chromosome 15 has been described in late S-phase of cycling human lymphocytes. Lymphocytes derived from PWS and AS patients with 15q11-13 deletions showed a deficiency in this pairing at the centromere (LaSalle and Lalande, 1996), demonstrating that homologous pairing of chromosome 15 is dependent on having both maternal and paternal copies of 15q11-13. Chromosome 15 is one of five human acrocentric chromosomes whose p-arms carry the genes for rRNA. These acrocentric chromosomes come together to form a single large nucleolus in mature neurons.

Evidence for homologous pairing of the imprinted 15q11-13 locus has been observed in the frontal cortex of human brain and in a differentiating human neuroblastoma cell line (Thatcher *et al.*, 2005). Using DNA FISH probes for *GABRB3* and the centromere of chromosome 15, a significant increase was observed in the homologous association of this region from infancy to adulthood in human frontal cortex that was not seen for another acrocentric chromosome. A similar increase in chromosome 15 homologous pairing was observed in the SH-SY5Y neuroblastoma cell line following induced differentiation in culture. Approximately 90% of all nuclei in the human frontal cortex exhibiting chromosome 15 pairing were large mature neuronal nuclei expressing high levels of MeCP2 (Fig. 2, unpublished data). Homologous 15q11-13 pairing was deficient in RTT, autism, PWS, and AS brain samples suggesting a similar pathway that is aberrant in all four disorders. Experimental evidence for the involvement of MeCP2 in 15q11-13 homologous pairing was obtained by transfecting the SH-SY5Y cells with an excess of a MeCP2 decoy, a methylated 22 bp DNA fragment which binds MeCP2, decreasing its binding to endogenous DNA within the nucleus. Homologous pairing was significantly blocked by the MeCP2 decoy, implicating MeCP2 in the mechanism (Thatcher *et al.*, 2005). Jugloff *et al.* (2005) showed that primary mouse cortical neurons over-expressing MeCP2 show increased axonal length and dendritic branching, perhaps due to altered chromatin conformation induced by higher levels of MeCP2 creating a nucleus transcriptionally poised for neuronal maturational changes.

The combination of aberrant MeCP2 expression in autism, PWS, AS, and RTT brain, decreased *UBE3A* and *GABRB3* expression in RTT and autism brain, and binding of MeCP2 to the PWS-IC provides a compelling link between MeCP2 and the imprinted region 15q11-13 and suggests a mechanism linking all of these disorders together.

VI. A Higher Order Model for MeCP2 in Regulating Gene Expression within 15q11-13

The findings supporting a role for MeCP2 in regulating the PWS/AS region and the *DLX* imprinted region have suggested that MeCP2 does not simply act in *cis* to repress methylated genes. Evidence for chromatin loop changes in *Mecp2*-deficient mouse brain in the *DLX* locus (Horike *et al.*, 2005) suggests that MeCP2 may form a major structural basis for chromatin loop formation. Higher order nuclear organization models have suggested areas of high transcriptional activity, so recruitment to these regions by MeCP2 and associated factors could have a major impact on the expression of genes such as *UBE3A*, *ATP10A*, *GABRB3,* and potentially *GABRA5* and *GABRG3*.

Although much work lies ahead to define the other binding sites for each parental allele across this 2–3 MB region, a speculative model is shown in Fig. 3A that diagrams how MeCP2 binding sites may serve to colocalize the promoters of *UBE3A*, *ATP10A*, and *GABRB3* with the imprinting control regions (ICR) on either the paternal or maternal chromosome (pink for maternal and blue for paternal ICR). Just as for the more simple loop structures of *H19* and *Igf2* (Murrell *et al.*, 2004), allele-specific methylation patterns would result in different chromatin loop structures on each parental allele causing *SNRPN* to be silent on the maternal and *UBE3A* and *ATP10A* to be reduced on the paternal chromosome. While the loss of MeCP2 is not sufficient to change the imprinted expression patterns, it could result in the inaccessibility of the promoters of *Ube3a* and *Gabrb3* to the ICRs, thus reducing expression on both parental alleles.

While the model in Fig. 3A would be consistent with the experimental results for both mouse and human, human 15q11-13 has the added complexity that it is on an acrocentric chromosome that is tightly linked to nucleolar organization and undergoes homologous pairing during neuronal maturation (Thatcher *et al.*, 2005; Fig. 2). The model in Fig. 3B therefore incorporates the nuclear and nucleolar changes that were discussed in the previous section. During neuronal maturational differentiation, MeCP2 expression increases and MeCP2-associated heterochromatic regions fuse and form around the outer perimeter of the larger nucleolus. As the p arms of chromosome 15 contain repeated rDNA genes, the 15q11-13 regions are recruited to this region as well as other acrocentric chromosomes. However, the 15q11-13 region has an additional "glue," perhaps MeCP2 itself or the combination of MeCP2 and other proteins or regulatory RNAs, that allows a tighter interaction than other acrocentric chromosomes (LaSalle and Lalande, 1996; Thatcher *et al.*, 2005). It is predicted that both alleles of *UBE3A* and *GABRB3* would benefit from this interaction simply by being brought into closer proximity to the ICR and active chromatin hub on the opposing chromosome. In this way, homologous pairing would be required for the optimal level of expression of *GABRB3* and *UBE3A* without invoking a

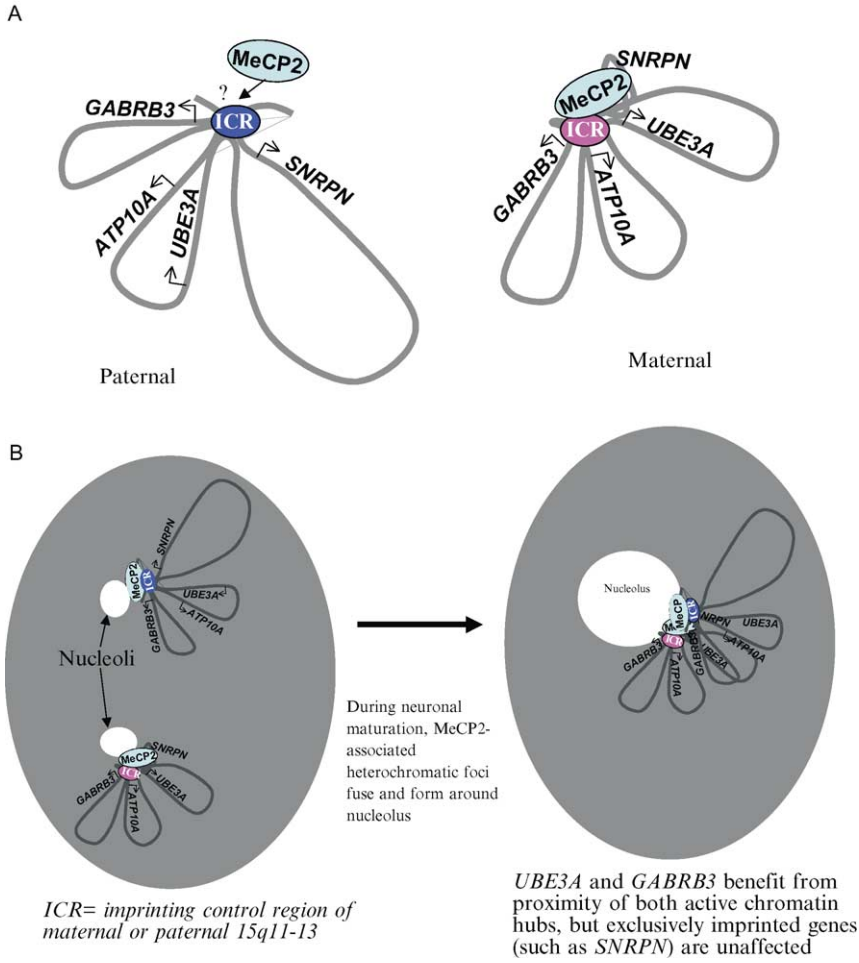

FIG. 3. A higher order model for MeCP2 in regulating gene expression within 15q11-13. (A) Chromatin loop structures are proposed that would change the gene expression patterns on the paternal and maternal 15q11-13 regions. Differential MeCP2 binding sites would be determined by differentially methylated regions (DMRs). One known binding site for MeCP2 is present in the maternal methylated allele of the *SNRPN* promoter, but presumably MeCP2 could also bind to sites of paternal-specific methylation. The chromatin loop structures could position the imprinting control regions (ICR) for either the paternal (PWS-ICR, blue) or maternal (AS-ICR, pink) ICR near the promoters of *Gabrb3* and *Ube3a*, thus explaining how MeCP2 deficiency can alter expression of these genes without necessarily binding at the promoters. (B) In the context of a neuronal nucleus undergoing maturational differentiation, the 15q11-13 chromatin loop structures are brought into close proximity by the formation of a large nucleolus (as in Fig. 2). MeCP2 colocalizes with the perinucleolar heterochromatin, bringing the associated chromatin loops together. *UBE3A* and *GABRB3* expression would benefit by being brought into the active chromatin hub of the opposite parental chromosome.

requisite change in the imprinted expression. The loss of MeCP2 function through mutation in RTT could result in reduced 15q11-13 pairing by a combination of aberrant chromatin looping of 15q11-13 and the loss of MeCP2-mediated interactions of homologous chromosomes. This model could also explain the more significant defects in UBE3A and GABRB3 expression in human RTT and autism samples compared to *Mecp2*-deficient mice (Samaco *et al.*, 2005), since syntenic mouse chromosome 7 does not show homologous pairing (Thatcher *et al.*, 2005).

The recent data showing autism brain samples having significantly reduced homologous pairing of 15q11-13 and reduced expression of *GABRB3* and *UBE3A* compared to age-matched controls raise interesting implications for understanding the etiology of autism. The samples examined also had significantly reduced levels of MeCP2, suggesting that the defects in pairing and 15q11-13 gene expression may be simply downstream effects of impaired neurodevelopment. Alternatively, the autism samples may have genetic or environmental factors reducing MeCP2 expression that could mimic the *MECP2* mutant state in RTT. Curiously, the autism samples showed more significant defects in homologous pairing than the RTT, AS, and PWS samples that have known genetic defects. In addition, the GABRB3 expression defects in autism were also more significant than those observed in RTT or *Mecp2*-deficient mouse brain. Could defects in other genetic and epigenetic pathways regulating GABRB3 expression be responsible for this finding? The linkage and association data using markers around *GABRB3* and within 15q11-13 are supportive of this possibility (Buxbaum *et al.*, 2002; Kim *et al.*, 2002; McCauley *et al.*, 2004; Nurmi *et al.*, 2001, 2003).

VII. Future Directions and Remaining Questions

Clearly, much more work lies ahead in understanding the epigenetics and genetics of gene regulation within 15q11-13 and its relevance to autism. Additional autism brain samples should be screened for expression defects and compared with normal controls and other neurodevelopmental disorders with known genetic etiologies with and without comorbid autism. First and foremost, the coding regions and upstream regulatory regions of *GABRB3*, *UBE3A*, and *MECP2* should be examined for genetic variants that may explain the expression defects in these genes in autism brain samples. Such genetic causes may be infrequent or heterogeneous in different autism families, as has been seen for the *NLGN* genes (Jamain *et al.*, 2003; Gauthier *et al.*, 2005; Vincent *et al.*, 2004). Alternatively, inheritance of polymorphic variants in or around these genes may be one of multiple susceptibility alleles that are predicted in the etiology of autism.

Further understanding of genetic and epigenetic pathways that regulate MeCP2 and GABRB3 expression is also expected to continue to shed light on

the molecular pathogenesis of autism. The MeCP2 binding sites and allele-specific methylation patterns across the entire 15q11-13 region need to be defined, as well as the proposed chromatin loop structures. Similar to the approaches used for determining chromatin loops in the relatively simpler regions of *H19/Igf2* and *Dlx5* loci (Horike *et al.*, 2005; Murrell *et al.*, 2004), the chromatin loops on both parental chromosomes across the entire 15q11-13 region could be defined. Although the imprinting status of transcription has been defined for much of the 15q11-13 region, the question of whether the $GABA_A$ receptor genes may show preferential allelic expression in human brain remains open. Perhaps proper expression of *GABRB3* depends on *trans* interactions between oppositely imprinted maternal and paternal 15q11-13 regions during homologous pairing that occur during neuronal maturation.

Mouse models are expected to continue to play an important role in understanding the syntenic chromosome 7qB4 region and the regulation of *Gabrb3* and *Ube3a*. Gene targeting knockouts of both genes have been made and could be useful in future experiments (Homanics *et al.*, 1997; Jiang *et al.*, 1998; Miura *et al.*, 2002). To truly mimic the expression level defects observed in autism, however, perhaps knock-down models should be attempted by transgenic or RNAi approaches. Testing the behavioral phenotype of these mouse models for relevance to autism would be important. In addition, testing the effect of double knock-down mice for *Mecp2* and *Gabrb3* or *Ube3a* may prove to be informative in understanding the combined effect of expression defects of these genes on behavioral phenotype.

Another potential avenue to pursue would be genetic and environmental interactions on MeCP2 and 15q11-13 gene regulation. As MeCP2 expression is tightly linked to neuronal maturation, pharmacological agents and other compounds that either enhance or reduce neuronal stimulation could affect MeCP2 expression levels. In addition, environmental agents such as mercury or pesticides that inhibit MeCP2 or GABRB3 function could be involved in triggering autism in genetically susceptible individuals.

In conclusion, we have discussed the results of the beginning stages of a "Rosetta stone" approach to understanding how distinct genetic defects may contribute to a common pathway for regulating nuclear organization, chromatin structure, and gene expression in the developing brain.

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GABAERGIC CEREBELLAR SYSTEM IN AUTISM: A NEUROPATHOLOGICAL AND DEVELOPMENTAL PERSPECTIVE

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Alterations in the GABAergic system in autism only recently became evident affecting specific receptors in the hippocampus, GAD protein levels in parietal cortex and cerebellum and a decreased number of GABAergic Purkinje cells evident in a subset of autistic cases. A candidate gene for the GABA-Aβ3 subunit has remained attractive on the chromosome 15q11-q13 region in a subset of individuals. Current studies are revealing valuable data toward understanding how cerebellar circuitry may be altered in autism as better markers and techniques become available. In particular, immunostaining of Purkinje cells demonstrated that previous Nissl studies may have overlooked agonal effects on the efficacy of tissue sections bringing into question results of previous studies, some of which also did not control for age. It appears that the cerebellum in autism shows variability in neuropathology similar to that described for other affected brain areas in autism. Decreased Purkinje cells are present in about half of cases in the posterior lateral cerebellar cortex yet normal cytoarchitecture persists throughout the cerebellum indicating completion of migration of both Purkinje cell and granule cell populations. Despite the Purkinje cell decrease, normal density of GABAergic interneurons, basket and stellate cells, are present in the molecular layer and neurons are also preserved in the principal olive that projects to the posterior lobe in the lateral hemisphere. This suggests that the Purkinje cell loss probably occurs at about 32 weeks of gestation or later but most likely no later that early postnatal development. Current studies have demonstrated that Purkinje cells are deficient in GAD67 possibly affecting GABA synthesis.

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Altered input to the deep cerebellar nuclei is discussed and how recent studies strongly link the cerebellum as a modulator of both motor and cognitive function thus potentially implicating it in a variety of autistic behaviors.

I. Introduction

The GABAergic system in autism has received increased attention in the recent literature largely due to: (1) reports of decreased numbers of cerebellar GABAergic Purkinje cells especially in the posterior lobe (Bailey *et al.*, 1998; Bauman and Kemper, 1985, 1994; Kemper and Bauman, 1998; Ritvo *et al.*, 1986; Whitney *et al.*, 2004); (2) decreased levels of key synthesizing enzymes (GAD65 and GAD67) in the cerebellum and parietal cortex (Fatemi *et al.*, 2002) and recently, decreased GAD67 localized to Purkinje cells (Yip *et al.*, 2005a); (3) neuropathology in the deep cerebellar nuclei, many cells of which are GABAergic (Bauman and Kemper, 1985, 1994; Kemper and Bauman, 1998); (4) decreased density of GABA-A receptors and benzodiazepine binding sites in specific subfields of the hippocampal formation (Blatt *et al.*, 2001) and increased packing density of parvalbumin-labeled GABAergic interneurons in the CA3 and CA1 subfields (Lawrence *et al.*, 2005); (5) the most common chromosomal abnormality in autism is an alteration(s) in chromosome 15q11-q13, a region that contains three GABA-A receptor subunit candidate genes for autism (Schroer *et al.*, 1998; Shao *et al.*, 2003) including the GABA-Aβ3 subunit receptor gene (Buxbaum *et al.*, 2002; Martin *et al.*, 2000; Shao *et al.*, 2003); and (6) elevated plasma GABA in autistic children aged 5–15 years (Dhossche *et al.*, 2002). This chapter will primarily focus on the cerebellar GABAergic system in autism since it has repeatedly been described by researchers as the most consistent neuropathological finding in autism. Caution should be taken however to make such a claim as more advanced techniques are redefining what we know about cerebellar pathology in autism. Similar to other neuropathological abnormalities in the autistic brain, results among cases are variable and not complete. This chapter will also address the functional implications of upsetting the delicate balance of excitatory-inhibitory inputs to key cerebellar GABAergic neurons as well as address the current and future path of investigation of this important inhibitory system in autism research.

II. The GABAergic System in the Cerebellum in Autism

A. Effects on Purkinje Cells

The initial study reporting reduced numbers of cerebellar Purkinje cells (PCs) in autism reporting on 4 cases age 3 to 33 years of age was by Williams *et al.* (1980). General PC loss was reported and most patients had histories of seizures.

Ritvo *et al.* (1986) reported the density of PCs in the cerebellar hemisphere and vermis of 4 autistic males, all without history of seizure activity, ranging in age from 10 to 22 years with a mean age of 17.5 years compared to a control group ranging in age from 3 to 13 years with a mean age of 7.75 years. The disparity in age of the two groups makes interpretation of these results difficult. These authors found significantly lower PC counts in the autistic cases but failed to account for the age-related change in cerebellar size (Hall *et al.*, 1975). Hall *et al.* (1975) showed that a normal reduction in the number of PCs per measured cortical distance normally occurs with age as the brain grows. For example, Hall *et al.* (1975) found that in males 10 years and under the mean PC count was 5.15 PCs/mm and ranged from 4.40 to 5.95 PCs/mm whereas males between the ages of 20 and 50 years had a mean PC count of 4.47 PCs/mm with a range of 3.40 to 6.92 PCs/mm. This difference could in part account for the lower PC count in the autistic group reported by Ritvo *et al.* (1986), as recalculation of Ritvo's data expressed in PCs/mm demonstrated that their findings are in agreement with Hall's reported normal variability in PC number with age (i.e., a mean of 5.6 PC/mm (range 5.0 to 6.3) in the younger group and a mean of 4.2 PC/mm (range 3.8 to 4.6) in the older group.

In other studies, nine autistic brains (aged 6–54 years) were serially sectioned by Kemper and Bauman with results reported in the 1980s and 1990s in a series of three papers (Bauman and Kemper, 1985, 1994; Kemper and Bauman, 1998). These authors reported qualitative reductions (some severe) of PCs in the posterior lateral cerebellar cortex with lesser effects in the adjacent archicerebellar cortex. Normal numbers of PCs were observed in the anterior lobe (Bauman and Kemper, 1985) and vermis (Bauman and Kemper, 1985; Kemper and Bauman, 1998). There was a pallor of staining in Nissl sections in the granule layer in four cases suggesting possible reduction or alteration in granule cells.

More recently, Whitney *et al.* (2004) quantified PCs in six autistic and four age-matched control brains in the posterior lateral neocerebellar cortex aged 13–54 years. With the investigator "blind" to the identification of the study groups, three cases with marked reductions in Nissl stained PCs (about 90% decrease) were identified. When the group code was broken, two of these three cases were controls! Further examination revealed that when the immunostain calbindin, a calcium-binding protein, was used on adjacent sections, the "missing" PCs in the 2 controls appeared but not in the autistic case! After extensive testing of Nissl stain and standard H & E preparation it was concluded that some brains must undergo agonal changes, even with short post-mortem intervals (PMI: the PMI in one case was only 5 hours) and these changes can directly affect the ability to count PCs using stereological techniques. When calbindin was used, 99^{+}% of PCs immunostained. Quantitative results revealed that three autistic cases had PC density in the normal range while the other three were reduced. Specifically, the calbindin PC counts in the control brains ranged from 4.0 to 5.4 PCs/mm with mean SD of 4.7 +/− 0.8 PCs/mm while cell counts in the autistic brains spanned a wider range, from 0.5 to 5.8 PCs/mm with

mean SD of 3.5 ± 1.8 PCs/mm (not significant by t-test, $p = 0.25$; Whitney *et al.*, 2004). Thus, reduction in PC density only appears to be in a subset of autistic brains and unless a specific immuno marker such as calbindin is used in conjunction with other stains the results may not be entirely accurate.

B. GAD Expression in Cerebellar Cortex is Altered in Autism

Glutamic acid decarboxylase (GAD) 65 and 67 kDa proteins, the key synthesizing enzymes for GABA, are reduced in both the cerebellar cortex and parietal cortex in autistic subjects (Fatemi *et al.*, 2002). In their study, cerebellar samples were taken from different cerebellar regions for Western Blot biochemical analysis, so results are difficult to interpret with regard to any one cerebellar area or which cell types might be affected. From seven young adult autistics aged 19–30 compared to nine age-matched controls, the mean GAD65 values in cerebellum were reduced by 51% and GAD67 reduced by 61%, both highly significant ($p < 0.02$ and $p < 0.03$, t-test respectively). Although the brain samples varied by location it was nevertheless an important finding because this suggests that the ability to synthesize GABA may be altered in the autistic cerebellum.

Yip *et al.* (2005a) addressed the issue as to whether it is the Purkinje cell that has a major reduction in GAD. In posterior lateral cerebellar cortex from 16 cases, eight autistics aged 16–30 years and eight controls aged 19–43 years, these investigators used *in situ* hybridization to measure GAD67 mRNA in a quantitative study. Yip *et al.* (2005a) found that there is a 40% decrease in GAD67 mRNA in PCs in the autistic cases relative to controls and this difference was found whether there was a decrease in PCs or not. Since every PC contains GAD67, PC counts were made in the same 16 cases and most autistic cases contained normal numbers of PCs per length of posterior lateral cerebellar cortex (i.e., most counts were in the low normal range). This provides further evidence that the PC decrease in autism is limited to a subset of cases, not a consistent neuropathological finding in autism and, that the majority of the GAD67 decrease reported in the cerebellum by Fatemi *et al.* (2002) is indeed found in Purkinje cells.

C. GABAergic Interneurons in Cerebellar Cortex in Autism

With the quantitative assessment of decreased cerebellar PCs in a subset of autistic cases, the question arises as to the state of GABAergic basket and stellate cells in the molecular layer of the cerebellar cortex. These interneurons intimately innervate PCs; the basket cells send horizontal axons in the lower third of the

molecular layer (parallel to the pial surface) forming axonal plexuses around the soma of each PC as a "nest." In contrast, stellate cells are present mainly in the outer two-thirds of the molecular layer and innervate PC dendrites. Both cell types immunostain with the calcium binding protein parvalbumin enabling quantitative density counts possible. Whitney *et al.* (2005) performed a stereological count in the posterior lateral cerebellar cortex in the same lobular location in the same 10 cases where PC counts were reported (Whitney *et al.*, 2004). In this way, the density of basket and stellate cells were calculated per PC from each control and autistic case. Results from the study demonstrated that there was no statistical difference in the density of either basket or stellate cells between control and autistic cases (i.e., even though there was moderate or severe PC loss in three out of six autistic cases, there was no change in GABAergic interneuron populations in the molecular layer) (Whitney *et al.*, 2005).

D. Implications of the Timing of Purkinje Cell Decrease in Autism

Another corollary observation is that in the autistic cerebellum, the adult laminar pattern of the cerebellar cortex is similar to controls (i.e., migration of the external granular layer is complete with no obvious ectopia). An exception may be PCs where Bailey *et al.* (1998) and colleagues have reported ectopic cells in autistic cases but these can also occasionally be found in controls. In any case, it is very difficult microscopically to tell the difference between an autistic and control cerebellar cortex, in any region or location, unless there is a decrease of PCs. If some Purkinje cells in autistic cases were never generated and thus did not migrate to their target location in the cerebellar cortex then it would be expected that there would be a major disruption in the cortical laminar pattern such as in the formation of the internal granular layer and, a likely loss of both granule cells and interneurons. Because the cerebellar cortical laminar pattern is not disturbed in autism, it is likely that PCs were generated, completed their migration to the PC layer, and then subsequently died. If the PCs died just after they established their position in the PC layer, before the elaboration of much of their dendritic tree, then it is likely that there would be a loss of interneurons, especially stellate cells, unable to make stabilizing synapses with their major targets. But because there is no difference in the density of GABAergic interneurons in the cerebellar molecular layer in autism, at least in the most affected area of reported PC loss, this infers that basket and stellate cells were able to make stabilizing synaptic contacts prior to the PC death. Therefore, from the results of Whitney *et al.*'s (2004, 2005) studies, the timing of the PC loss in autism is most likely not before 32 weeks of gestational age but may extend into early postnatal development since the completion of migration of the external granular layer in humans is not complete until about 18–20 months.

Bauman and Kemper (1985) reported a pallor of staining in the granular layer in some but not all of their original eight cases. It could be that in those cases, the Purkinje cell loss may have occurred earlier rather than later in this range (i.e., late prenatal period affecting the survival of some granule cells whereas cases with normal Nissl stained granule cells but with PC loss may represent a loss in the early postnatal period). Although this is speculation, there is additional evidence that suggests PC loss during this approximate time period and that is the preservation of inferior olivary neurons that project excitatory olivocerebellar climbing fibers directly to Purkinje cells.

In a stereological study of adult autistic and control brainstems, Thevarkunnel *et al.* (2005) reported normal numbers of neurons in the principal olive in autistic cases, the subnucleus that directly projects to the lateral cerebellar hemisphere including the posterior lobe. Again, the preservation of inferior olivary neurons, similar to the GABAergic interneruons in the molecular layer, have in common that their primary target neuron is the Purkinje cell. Olivocerebellar climbing fibers (CFs) arrive early in cerebellar development when PCs are still in clusters awaiting final migration to the PC layer (Sotelo *et al.*, 1984). Permanent stabilizing synapses are made when PCs are in their final position and CF continues to grow as the PC elaborates its extensive dendritic arbor in a mostly two-dimensional plane. The formation of the olivocerebellar projection is thought to be regulated by positional information shared by pre- and post-synaptic neurons. Sotelo and Chedotal (1997) suggested the cell adhesion molecule BEN/SCI/DM-GRASP, isolated from chick embryos, could be one of the target recognition molecules controlling the development of the olivocerebellar projection to innervate its target PC thus forming a topographic map. Postnatal loss of PCs in spontaneous genetic mutations in mice results in inferior olivary cell loss as a secondary consequence with some cells surviving probably due to their sustaining collaterals to the deep cerebellar nuclei (Blatt and Eisenman, 1985a,b). In mutant mice, the PC loss is usually regional and continuous along folia and each CF innervates only two to four PCs increasing the likelihood that a particular CF would lose all of its intended targets in the PC layer. In marked contrast, each CF in the human innervates up to ten PCs and the PC decrease observed in autism results in clusters of remaining PCs (Bailey *et al.*, 1998; Whitney *et al.* 2004, 2005) with the clusters variable from section to section (Whitney *et al.*, 2004, 2005). Therefore, the likelihood of all intended target PCs missing for a given CF is low presumably contributing to the survival of the inferior olivary cells. The timing of events allowing this survival is likely at a similar temporal period as the elaboration of the PC dendritic tree (i.e., between 32 weeks prenatally into the early postnatal period). Interestingly, one case that Whitney *et al.* (2004, 2005) counted had a severe PC loss in the posterior lateral cerebellar cortex (95% decrease) but had a normal number of principal olive cells (Thevarkunnel *et al.*, 2005) although the counts were in the low normal range. This suggests that if the PC loss is large enough some inferior olivary cells

may also die but not in sufficient numbers to place it out of the low normal range. It is unknown whether a contributing factor to inferior olive cell survival in autism is their sustaining collaterals to the deep cerebellar nuclei (DCN) because there is a report in the literature that these nuclei also undergo neuropathological changes in autism (Bauman and Kemper, 1985, 1994; Kemper and Bauman, 1998).

E. Neuropathology of the Deep Cerebellar Nuclei in Autism

Age-dependent changes were observed in the deep cerebellar nuclei (Bauman and Kemper, 1985, 1994; Kemper and Bauman, 1998) such that brains from young autistic individuals exhibited unusually large neurons in all four DCN with older individuals (>22 years) showing unusually small and pale neurons with qualitative observation of diminished neuronal numbers in the fastigial, globose, and emboliform nuclei. It must be stressed that as of the date of this publication, there have not been any quantitative studies of the DCN in autism so it is unknown how the PC loss in some autistic cases affects cell number in the DCN. From qualitative observations, the dentate nucleus appears the least affected (Bauman and Kemper, 1985), which is surprising since one of its main inputs is from PC axons emanating from the posterior lateral hemisphere, the area considered most affected with decreases of PCs. The presumed preservation of dentate neurons may indicate a reorganization of PC inputs and/or CF collaterals. Any disruption in the delicate inhibitory-excitatory balance of inputs to either PCs or DCN cells potentially can have profound consequences to the output of the DCN and presumably affect functionality of the nucleo-olivocerebellar system and/or the dentatorubrothalamic tract (Brodal, 1978) projecting back to cerebral cortical areas. Some DCN cells are GABAergic and it has been demonstrated by de Zeeuw *et al.* (1994, 1997, 1998) that these inhibitory neurons project back to the inferior olive to modulate its activity. Since firing of inferior olivary neurons is synchronous via coupled electrical synapses, disruption in the GABAergic feedback of the DCN could potentially interfere with the ability of inferior olivary neurons to generate coherent rhythmic outputs thus slowing overall cognitive processing speed (Welsh, 2002; Welsh *et al.*, 2005). Alterations within this circuit due to possible miswiring (heterotopic innervation), hyperinnervation of CFs to its PC and/or DCN targets, or innervation of fewer targets due to cell loss would also have the potential of disrupting the modulatory effects of cerebellar function.

III. Functional Consequences of Altered Cerebellar Function in Autism

The classical literature has described the main function of the cerebellum as a motor structure, modulating the output of motor cortex to produce fine motor coordination and skills. Although there are motor signs in autism such

as repetitive and stereotypic movements of the body, limbs and fingers, hyperactivity, abnormal muscle tone and reflexes, and usual gait patterns (see Bauman, 1999, for comprehensive review), autistic individuals do not demonstrate classic cerebellar signs such as marked ataxia and it has thus been difficult for investigators to correlate PC deficits or DCN alterations with the severity of autism or to specific autistic behaviors based only on the consideration of motor criteria.

In normal children, cerebellar hemisphere tumor resection yields a quite different pattern of clinical deficits. Children demonstrated impairments in executive functions, auditory and visual learning, time-based attention tasks, expressive language, and visuospatial abilities (Levisohn *et al.*, 2000; Riva and Giorgi, 2000). Clinical research indicates that discrete cerebellar lesions, in otherwise healthy children, cause behavioral and/or cognitive impairments. In autism, however, cerebellar pathology is likely acquired during critical developmental period(s), during a time when the brain is capable of constructing alternate innervation patterns (Sugihara *et al.*, 2003; Zagrebelsky *et al.*, 1997). The apparent lack of correlation of clinical and neuropathological data may be secondary to severe behavioral problems in some individuals that impede testing of cognitive functions attributable to the cerebellar damage. Future research elucidating the correlation between cerebellar pathology and autism phenotype will likely require consistent and detailed neuropsychological testing of individuals with autism.

What are the neuroanatomical substrates within the cerebellar circuitry that may in part underlie cognitive changes observed in the autistic phenotype? Much progress has recently been made in tract tracing and lesion-behavior studies in the rhesus monkey. In a large number of manuscripts, Schmahmann and Pandya (1997; also see Schmahmann, 2001a,b, for review) mapped out the cerebral cortical projections to the cerebellar cortex in the rhesus monkey and found that the pons receives widespread afferents from prefrontal, posterior parietal, parastriate, and limbic cortices in a highly organized pattern. In turn, segregated loops of cerebellar-thalamo-cortical projections have the potential of participating in a wide variety of motor, cognitive, and emotional behaviors, in a diverse stream of information flow. This new revolutionary view of cerebellar function actually has its roots in Ito's theories (see Ito, 1993, for review) but has been largely overlooked. In autism, it is more likely that cerebellar abnormalities may better correlate with functional and high order behavioral alterations rather than classical motor disturbances. We are in the early stages in deciphering how the cerebellum works contrary to Eccles *et al.*'s (1967) original view that the simplicity of its cytoarchitecture and simple physiological characteristics would make it one of the first understood structures in the human brain. Within the simple cytoarchitecture, however, lies very organized streams of information processing affecting a diverse number of sensorimotor, associative, and limbic functions.

Schmahmann *et al.* (2004) utilized MRI targeted electrolytic lesions of the dentate nucleus bilaterally in rhesus monkeys and assessed them on conceptual set shifting tasks, a modified version of the Wisconsin card sorting task. This test determines the monkey's ability to learn a rule based on abstract principles and then to "shift set" as the rule shifts from one concept condition to another. Statistically significant greater perseveration was found when the monkeys' shifted from one abstraction to another. This test has been shown to be sensitive to prefrontal cortical damage, and suggests that the cerebellum is a critical modulator of prefrontal systems mediating executive function (Schmahmann *et al.*, 2004). In autism, altered GABAergic PC input to the DCN seen in at least half of examined cases with decreased numbers of PCs, would have direct effects on the normal functioning of the DCN and thus may be an underlying substrate, perhaps among many, that may contribute to the behavioral phenotype.

IV. Future and Ongoing Neuropathological Experiments to Further Understand How Cerebellar Circuitry is Altered in Autism

First, a detailed examination of the pontine nuclei in autistic individuals is needed to determine whether the "motor" or "cortical association" input region(s) are affected either in cell number, cell size, and/or distribution. This study should be correlated with a detailed analysis of the cerebellar granular layer. Next, olivocerebellar CFs that use glutamate and aspartate as neurotransmitters need to be marked to determine whether its distribution their altered in the cerebellar cortex especially in those cases with decreased numbers of Purkinje cells. In our laboratory, Yip *et al.* (2005b) is using the neurofilament marker peripherin specific to CFs and to two mossy fiber pathways, spinocerebellar and vestibulocerebellar tracts. She found that CFs do indeed innervate PCs and extend into the molecular layer in autistic cases and noticed some differences in the thickness and pattern of CF distribution to PCs and in its collaterals to the dentate nucleus (Yip *et al.*, 2005b). Another study underway in our laboratory is determining the density and distribution of GABA receptor types in the cerebellar cortex and dentate nucleus. This should provide important insights into the functionality of the GABAergic system especially since there is reduced GAD67 mRNA in Purkinje cells (Yip *et al.*, 2005a). Finally, a detailed quantitative analysis of all four deep cerebellar nuclei is needed to determine the extent of any cell loss and investigate the possible cause(s) of the age-related change in cell size first recognized by Bauman and Kemper (1985). Indications thus far suggest that cerebellar circuitry is disrupted in autism possibly at many levels and pathways. Attention to correlates with altered cognitive functions should lead to a better understanding of the role(s) of the cerebellum in autism. Identification of candidate genes in autism

may also yield much needed information regarding the developmental timing of events influencing connectivity. Autism may be the result of action of many genes acting at different but precise times during pre- and early postnatal development in the cerebellum and at other sites in the brain.

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REELIN GLYCOPROTEIN IN AUTISM AND SCHIZOPHRENIA

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Reelin glycoprotein is a serine protease with important roles in embryogenesis and in adult life. Reelin mutations or deficiency of the protein product could cause abnormal cortical development and/or Reelin-induced signal transduction impairment in brain. Reelin abnormalities in several neuropsychiatric disorders, such as autism and schizophrenia, may provide mechanistic explanations for etiologies of these disorders.

I. Reelin Gene and Protein

Reelin glycoprotein is a serine protease (Quattrocchi *et al.*, 2002) with dual roles in mammalian brain: embryologically, it guides neurons and radial glial cells to their final positions in brain (Forster *et al.*, 2002; Goffinet, 1992); during adult life, it participates in a signaling cascade which may serve synaptic plasticity, memory processing, and cognition (Fatemi, 2005; Weeber *et al.*, 2002). Reelin gene (Reln) is localized to chromosome 7 in man (DeSilva *et al.*, 1997). The Reln gene codes for protein products which on SDS-PAGE range from 410 to 330, 180kDa and several smaller fragments in man (Fatemi *et al.*, 2002a, 2004, 2005a; Ignatova *et al.*, 2004; Smallheiser *et al.*, 2000) and in rodents (D'Arcangelo *et al.*, 1995; Ogawa *et al.*, 1995).

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II. Reelin Signaling Cascade

Reelin protein binds several receptors including apolipoprotein E receptor 2 (ApoER2), very-low-density lipoprotein receptor (VLDLR), and $\propto 3\beta 1$ integrin to initiate a signaling cascade which underlies downstream biochemical events leading to synaptic plasticity (see Fig. 1). Binding of Reelin to its receptors, specifically ApoER2 and VLDLR, induces clustering of these receptors and oligomerization of the adaptor protein, disabled-1 (Dab-1) (D'Arcangelo *et al.*, 1999; Dulabon *et al.*, 2000; Hiesberger *et al.*, 1999; Strasser *et al.*, 2004). This is then followed by tyrosine phosphorylation of Dab-1 which causes actin polymerization and final ubiquitination of Dab-1 protein, steps involved in mechanisms behind cell migration and synaptic plasticity (Beffert *et al.*, 2005; Suetsugu *et al.*, 2004). Phosphorylation of Dab-1 can also act as a substrate for inhibition of the level of glycogen synthase-kinase 3β (GSK-3β) and modulation of pathways for cell survival and growth (Beffert *et al.*, 2002).

III. The Reeler Mouse

The significance of Reelin's role in embryogenesis of brain became evident following the discovery of a Reln gene mutation nearly half a century ago (Falconer, 1951). The Reelin mutant mouse, which carries an autosomal recessive mutation in Reln, exhibited ataxia and a reeling gait. Examination of the brain in these animals showed inverted cortical lamination, abnormal positioning of neurons, and aberrant orientation of neuronal cell bodies and nerve fibers (Falconer, 1951; Goffinet, 1979). The mutant mice also exhibited cerebellar hypoplasia with associated lack of foliation (Caviness and Sidman, 1973). Ectopic expression of Reelin in the Reeler mouse rescues cerebellar development and corrects ataxia in the mutant mouse (Magdaleno *et al.*, 2002). The homozygous mutant mouse does not produce Reelin. The heterozygous Reeler mouse has a 50% reduction in Reelin protein and mRNA with decreases in dendritic spine density, neuropil hypoplasticity, and decreased GABA turnover (Carboni *et al.*, 2004). Behaviorally, the heterozygous Reeler mouse exhibits decreased prepulse inhibition, a phenomen observed in subjects with autism and schizophrenia (McAlonan *et al.*, 2002; Meincke *et al.*, 2004; Tueting *et al.*, 1999). Several recent reports using various prenatal insults, e.g., viral infection in midterm pregnant mice (Fatemi *et al.*, 1999, 2002b), and 5 methoxytryptamine exposure in E17 pregnant rats (Janusonis *et al.*, 2004) cause reductions in levels of brain/blood Reelin levels and result in abnormal corticogenesis in the offspring.

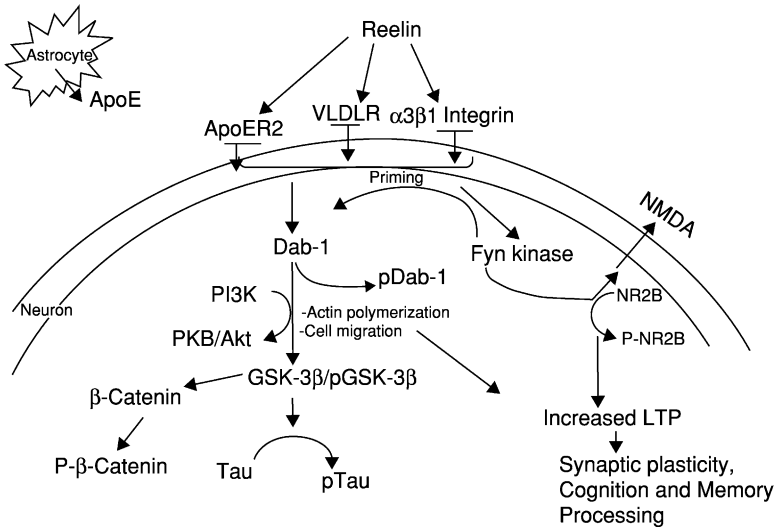

FIG. 1. The Reelin signaling system and cognition. Extracellular Reelin glycoprotein is secreted by Cajal-Retzius cells and certain cortical and hippocampal GABAergic cells and cerebellar granule cells. Reelin can bind its receptors ApoER2, VLDLR, and $\alpha 3\beta 1$ integrin directly, initiating the signaling system in the effector cells (i.e., cortical pyramidal cells). Reelin induction of the cascade leads to clustering of the receptors causing dimerization/oligomerization of Dab-1 protein and activation of Src-tyrosine kinase family/Fyn-kinase leading to tyrosine phosphorylation of Dab-1 protein in a positive-feedback loop. Interaction between Dab-1, N-WASP, and ARP 2/3 complex causes formation of microspikes or filopodia which are important in processes of cell migration and synaptic plasticity. Finally, phosphorylation of a subpopulation of Dab-1 molecules causes degradation of Dab-1 via ubiquitination, resulting in termination of Reelin signaling cascade. Downstream effector proteins involved in Reelin signaling path include phosphatidylinositol-3-kinase (PI3K) and protein kinase B (PKB/Akt), which further impact on three other important molecules, glycogen synthase kinase (GSK-3β), β-catenin, and tau. The latter proteins can modulate pathways, affecting cell proliferation, apoptosis, and neurodegeneration respectively. Finally, Reelin has a direct effect on enhancement of long term potentiation (LTP), via direct involvement of its receptors VLDLR and ApOER2. Alternately, tyrosine phosphorylation of NR2B subunit of NMDA receptor by Fyn kinase is essential for induction of LTP and modulation of synaptic plasticity, potentially converging on Reelin's role in cognition and memory processing (Fatemi *et al.*, 2001, 2005b).

IV. Reelin in Autism

Analogous defects involving the Reelin signaling system appear to be present in several neuropsychiatric disorders (e.g., schizophrenia (Fatemi *et al.*, 2000, 2005a; Guidotti *et al.*, 2000; Impagnatiello *et al.*, 1998) and autism (Fatemi *et al.*, 2001, 2005b)). The findings of Reelin defects are more robust in autism and are supported by two positive genetic linkage studies (Persico *et al.*, 2001;

TABLE I
mRNA Levels for Reelin, VLDLR, Dab-1, and GSK3*

Area	Gene of interest (GOI)	GOI relative to age matched control	P Value
Area 9	Reelin	−4.7	$p < 0.035$
	VLDLR	+14.2	$p < 0.01$
	DAB1	−5.4	$p < 0.01$
	GSK3	+1.9	NS
Cerebellum	Reelin	−3.9	$p < 0.01$
	VLDLR	+2.8	$p < 0.04$
	DAB1	−3.4	$p < 0.001$
	GSK3	+1	NS

*Fatemi, S. H., *et al.* (2005b).

Zhang *et al.*, 2002). Autism, which is a severe childhood disorder of the brain, is characterized by impairments in communication, social skills, and repetitive behavior (Kanner, 1943). Despite a large body of evidence supporting a genetic cause for autism (Folstein and Rosen-Sheidley, 2002), environmental causes are potentially responsible for some cases of autism (Rodier, 2000). A controversial issue is the involvement of Reln in causation of autism. Of the six genetic linkage studies, four deny a link (Bonora *et al.*, 2003; Devlin *et al.*, 2004; Krebs *et al.*, 2002; Li *et al.*, 2004) while two are supportive (Persico *et al.*, 2001; Zhang *et al.*, 2002). Additionally, four recent biochemical reports, however, show reductions in brain (Fatemi *et al.*, 2001, 2005b) and blood (Fatemi *et al.*, 2002a; Lugli *et al.*, 2003). Reelin levels in subjects with autism (Fatemi *et al.*, 2005b) showed that Reelin protein and mRNA species were reduced significantly in area 9 and cerebellum of autistic subjects vs. age and postmortem intervaled-matched controls (see Table I). The reductions in Reelin levels accompanied significant increases in mRNA levels of Reelin receptor VLDL-R in frontal and cerebellar cortices of autistic subjects (Table I). Surprisingly, levels of Dab-1 mRNA were also reduced significantly in the same brain sites in autistic subjects (Table I) implying involvement of the Reelin signaling cascade in the autistic pathology (see Fig. 2).

V. Reelin in Schizophrenia

Schizophrenia, a neurodevelopmental disorder, which in contrast to autism affects youth in puberty and is manifested by presence of hallucinations, disorganized behavior, and fragmentation of thought (Kraeplin, 1923), may also share

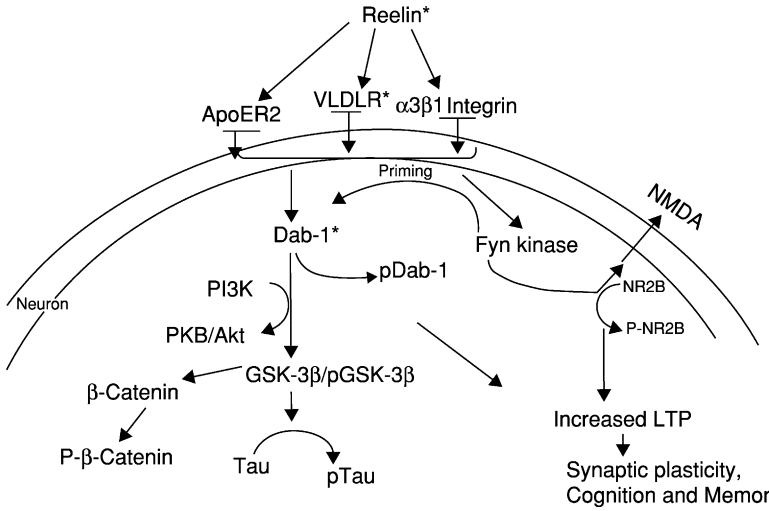

FIG. 2. The role of Reelin signaling system in autism. Normally, extracellular Reelin is secreted by Cajal-Retzius cells and certain GABAergic cells to bind its receptors VLDLR, ApoER2, and $\alpha 3\beta 1$ integrin on effector cells. Following binding of Reelin to its receptors, Dab-1 protein is oligomerized and phosphorylated. In autistic brain, Reelin signaling system appears to be impaired in 3 steps (marked by *); (1) Reelin ligand is not produced adequately as evident by reductions in mRNA and protein levels in superior frontal cortex and cerebellum; (2) Reelin receptor VLDLR mRNA is upregulated potentially in response to reduced levels of its ligand, Reelin; (3) Dab-1 mRNA is also reduced potentially due to reduction in levels of Reelin which normally activates Dab-1 phosphorylation via a positive-feedback loop. Alternatively, Dab-1 levels may be reduced in response to increases in levels of VLDLR acting via a negative-feedback loop. Alterations in levels of Reelin, its receptor VLDLR, and adaptor protein Dab-1 interfere with the Reelin signaling system affecting LTP, synaptic plasticity, cognition, and memory, modalities involved in autism (Fatemi *et al.*, 2005b).

Reelin abnormalities with autism (Fatemi, 2005). Investigation of the postmortem brains using a multitude of techniques showed downregulation of Reelin protein and mRNA in the prefrontal cortex (Guidotti *et al.*, 2000), and decreased Reelin protein in hippocampus (Fatemi *et al.*, 2000) and cerebellum (Fatemi *et al.*, 2005a) of subjects with schizophrenia. Mechanistically, it appears that hypermethylation of the promoter for Reln may be partially responsible for the observed decreases in Reelin in schizophrenia (Abdolmaleky *et al.*, 2005; Costa *et al.*, 2003). Two genetic linkage studies have not been able to show a significant linkage between Reelin polymorphisms and schizophrenia (Akahane *et al.*, 2002; Chen *et al.*, 2002). The biochemical data also support involvement of Reelin abnormalities in mood disorders (Fatemi *et al.*, 2000, 2005a; Guidotti *et al.*, 2000) regardless of presence of psychosis.

VI. Conclusions

In summary, increasing biochemical evidence points to involvement of Reelin glycoprotein in a number of psychiatric disorders including schizophrenia, autism, and mood disorders. The disparity seen in levels of Reelin production in man appears to be similar to the same scenario seen in various animal models which affect Reelin production, leading to production of cognitive deficits in rodents (Fatemi, 2005). Future correlative studies of human postmortem brains in schizophrenia and autism and pertinent animal models of mental disorders may expand our knowledge of the role of Reelin in cognition.

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IS THERE A CONNECTION BETWEEN AUTISM, PRADER-WILLI SYNDROME, CATATONIA, AND GABA?

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Research advances in autism, Prader-Willi syndrome, and catatonia allow new inferences about their mutual relations. Catatonia has been described in both people with autism and Prader-Willi syndrome, but remains unaccounted. Although autism and Prader-Willi syndrome have been considered entities in their own right, clinical observations suggest common ground and point to GABA dysfunction as a putative shared risk factor. The knowledge base on GABA abnormalities in autism, Prader-Willi syndrome, and catatonia is limited but expanding, and is summarized in this chapter. Preliminary findings that plasma GABA is a familial marker of autism are presented. Future research avenues are outlined.

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I. Introduction

> The interest in neurosis sparked by psychoanalytical ideas and the segregation of the "feeble-minded" into special institutions may be the potent factors in the decline of catatonia. Likewise, the impact of Kanner's delineation of autism, and research into the stereotypies of mental retardation has tended to mute the earlier enthusiasm concerning the interrelationship between the functional psychoses and mental retardation. In fact, the clinical and research "hole" resembles a form of diagnostic sterilization reminiscent of the operative practices of the eugenics period. Reviewing the whole process in the light of critical science and historical understanding in services shows up areas of underdevelopment in services, research, and clinical acumen. Yet the potential for genuinely new discoveries is there provided we understand what really has gone before.
>
> T. Turner, M.D. (1989)

Historically, autism, childhood schizophrenia, mental retardation, and other early-onset developmental disorders were thought to be connected (Earl, 1934; Turner, 1989). In 1943, Kanner isolated autism from other childhood disorders. This may have caused premature closure of the search for commonalities between autism, psychosis, mental retardation, and other early-onset neurodevelopmental disorders. However, research advances in these disorders offer an opportunity for re-evaluation. New findings reveal phenomenological, biochemical, and genetic areas of overlap. GABA dysfunction may provide a common risk factor in autism, Prader-Willi syndrome, and catatonia (Dhossche, 2004). In this chapter, evidence that GABA abnormalities are present in autism, Prader-Willi syndrome, and catatonia is reviewed. Implications for future research are described.

II. GABA in Autism

> The charm of GABA lies in nature's choice of this simple molecule, made from the common metabolic soil of glutamic acid, for the all-important role as major controller of the infinitely complex machinery of the brain, allowing it to operate in the manner best described as freedom without license. Try as one might, one cannot come up with a better choice for the job.
>
> Eugene Roberts (2000)

There are many theories about putative core deficit(s) in autism, but no definitive findings. Many psychological, physiological, biochemical, immunological, and genetic differences between autistic people and control groups have been reported (Rapin & Katzman, 1998). Some differences have been replicable; others have been attributed to the cognitive deficits that are prevalent in autistic people (Rutter, 1983). No comprehensive theory on autism has been clearly articulated in which psychomotor deficits play a central role, with the possible

exception of Robin Allott's contribution (2001) linking autism and the Motor Theory of Language.

GABA hypotheses of autism have recently been formulated (Dhossche *et al.*, 2002; Hussman, 2001). Hussman (2001) speculates that autism reflects dysfunction in a single factor (i.e., decreased GABA inhibition, shared in common by many systems). In his model, suppression of GABAergic inhibition results in excessive stimulation of glutamate specialized neurons and loss of sensory gating. Inhibition of GABA may become defective through multiple etiological factors. Loss of inhibitory control may cause deterioration in the quality of sensory information due to the failure to suppress competing "noise," resulting in compensatory restrictions in sensory input to a narrow, repetitive, or controllable scope.

Criteria for a comprehensive theory of autism have been formulated (Dhossche *et al.*, 2002). Any viable theory must consider the protean nature of symptoms, course, and outcome in autistic people, and should account for: (a) the early onset of clinical abnormalities; (b) worsening of symptoms around puberty in a considerable number of patients; (c) association between autism and epilepsy; and (d) genetic transmission of the disorder.

The evidence that central GABA dysfunction can account for these key features is briefly reviewed:

a. GABA is the main inhibitory neurotransmitter in the mature brain as perhaps 25–40% of all terminals contain GABA. In early development, GABA has an excitatory trophic role affecting neuronal wiring, plasticity of neuronal network, and neural organization (Roberts, 2000). Interference with the trophic role of GABA may affect development of neuronal wiring, plasticity of neuronal network, and neural organization (Christie *et al.* 2002; Herlenius and Lagercrantz, 2001; Varju *et al.*, 2001). For example, in mice, developmental changes in inhibitory synaptic currents in cerebellar neurons are determined primarily by developmental changes in $GABA_A$ receptor subunit expression. Overall, the effects of abnormal trophic GABA function could account for the brain abnormalities reported so far in autistic people (Courchesne *et al.*, 1988, 2001).

b. Decreased GABA inhibition in the hypothalamus is considered an important trigger for onset of puberty (Genazzani *et al.*, 2000; Mitushima *et al.*, 1994). Adaptive changes in GABA function at the onset of or during puberty may worsen or induce disorders associated with underlying abnormalities of GABA function. Increased rates of seizure disorders (Deykin and MacMahon, 1979; Gillberg, 1991b), catatonia (Wing and Shah, 2000), and worsening of autistic symptoms or overall behavioral deterioration (Gillberg, 1991a; Gillberg and Schaumann, 1981) have been reported in autistic people around and after puberty.

c. GABA has been strongly implicated in epilepsy (Petroff *et al.*, 1996). About 30% of autistic people develop some type of epilepsy (Gillberg, 1991b).

d. Genetic studies have implicated the proximal long arm of chromosome 15 in autism (Bakker *et al.*, 2003; Borgatti *et al.*, 2001a; Cook *et al.*, 1997; Lauritsen *et al.*, 1999) and catatonia (Stöber *et al.*, 2000, 2002). Three $GABA_A$ receptor subunits genes (GABRB3, GABRA5, and GABRG3) are located at the chromosome location that includes the PWS/AS region. Animal and human studies have suggested a role for these genes in the phenotypic expression of Prader-Willi syndrome (Ebert *et al.*, 1997) and Angelman syndrome (DeLorey *et al.*, 1998; Odano *et al.*, 1996). GABRB3 has been associated with autism in several studies (Cook *et al.*, 1998; Menold *et al.*, 2001; Nurmi *et al.*, 2003), especially in patients with increased levels of "insistence on sameness" (a composite score of difficulties with minor changes in personal routine or environment, resistance to trivial changes in environment, and compulsions/rituals) (Shao *et al.*, 2003) and savant skills (Nurmi *et al.*, 2003).

Empirical support for GABA dysfunction in autism is limited. Elevated plasma GABA levels in autistic children were found in a case-report (Cohen, 1999) and case-series (Dhossche *et al.*, 2002). Reduced ^{3}[H]-flunitrazepam labeled benzodiazepine bindings sites and ^{3}[H]-muscimol labeled $GABA_A$ receptors have been reported in the hippocampus of autistic people (Blatt *et al.*, 2001), providing direct evidence of abnormal benzodiazepine-GABA receptor complexes in this brain region. Glutamic acid decarboxylase (GAD) is the enzyme responsible for normal conversion of glutamate to GABA in the brain. GAD exists in two isoenzymes, GAD_{65} and GAD_{67}, that are products of two independently regulated genes (Soghomonian and Martin, 1998). In a postmortem study (Fatemi *et al.*, 2002), brain levels of both isoenzymes were reduced approximately by 50% in the parietal and cerebellar cortices of autistic patients. GAD deficiency in autism may be due to or associated with brain abnormalities in levels of glutamate/gamma amino butyric acid, or transporter/receptor density.

III. Plasma GABA as Marker for Autism

GABA is present in plasma at very low concentrations. Its role is unknown. Its correlation with GABA in brain or peripheral organs is also not clear. There is evidence that plasma GABA levels are to some extent under genetic control (Petty *et al.*, 1999). Others have observed that blood plasma levels seem fairly constant among various mammalian species (i.e., between 500–1200 pmoles/ml) (Ferkany *et al.*, 1978). This suggests non-dietary sources of plasma GABA.

Plasma GABA levels may reflect global or local aspects of brain GABA activity. However, there are no studies that have correlated plasma GABA levels and localized brain GABA levels. A peripheral source of plasma GABA has been

postulated but has not been demonstrated, at least not in normal physiological conditions (Petty *et al.*, 1987). GABA does not enter the brain easily through the brain blood barrier (van Gelder and Elliot, 1958). GABA is eliminated from the brain interstitial fluid to the circulating blood across the brain blood barrier (Kakee *et al.*, 2001). This efflux mechanism is inhibited by probenecid and valproic acid (Löscher and Frey, 1982). There is active transport of GABA into the CSF (Löscher and Frey, 1982).

Relations between plasma, CSF, and brain GABA levels are unclear. Correlations between plasma and CSF GABA levels and correlations between changes in plasma and CSF GABA levels under specific pharmacological conditions (Ferkany *et al.*, 1978; Löscher and Schmidt, 1981) or (patho)-physiological states (Adinoff *et al.*, 1995) have been reported in some studies, but not all (Berrettini *et al.*, 1982; Schmidt and Löscher, 1982).

The simultaneous assessment of levels of brain, CSF, and plasma GABA levels in future studies may clarify their correlations in different disorders and under different conditions. However, it cannot be automatically assumed that central GABA measures (CSF and brain) perform better than plasma GABA levels as a marker of GABAergic function in specific disorders. This has been demonstrated in a study in schizophrenia (van Kammen *et al.*, 1998) where plasma GABA levels, but not CSF GABA levels, correlated with specific brain morphology.

There are a few studies that have measured plasma GABA in human diseases. Low levels of GABA were found in CSF of patients with Alzheimer's disease (Jimenez-Jimenez *et al.*, 1998) and in plasma of patients with bipolar disorder (Petty *et al.*, 1993) and mood disorders (Petty *et al.*, 1992). Low post-trauma GABA plasma levels have been suggested as a predictive factor in the development of acute posttraumatic stress disorder (Vaiva *et al.*, 2004). Elevated GABA levels were observed in a small sample of autistic children (Dhossche *et al.*, 2002).

Measuring plasma GABA measurements is difficult because of the low concentrations and the need to process samples quickly. It has been estimated that about 30% of whole blood GABA is present in plasma (Ferkany *et al.*, 1978). The remaining fraction is bound to formed elements, mostly homocarnosine i.e., a dipeptide. CSF GABA increases during storage at room temperature, probably because of GABA release from homocarnosine (Grossman *et al.*, 1980).

A sensitive assay is critical for adequate measurement of plasma GABA. In the past, various methods have been developed for the determination of GABA. These methods were based on techniques including HPLC, GC-MS, CE-MS, CE/laser induced fluorescence detection, electrochemical sensor, and spectrophotometry. Most of these methods, particularly the GC-MS based methods, required time-consuming sample pretreatments such as liquid-liquid or solid phase extraction. HPLC coupled to mass spectrometry (MS) with an electrospray ionization (ESI) source has become a popular analytical technique. However, the sensitivity of an HPLC-MS combination is often lackluster compared with

fluorescence detection, particularly when standard-size HPLC columns (4mm ID) are used. This problem can be simply caused by sample dilution within the relatively large volume comprised by the column and tubing. Therefore, capillary HPLC-MS hyphenation is gaining research interest.

Our group has developed a sensitive HPLC-ESI-MS/MS method for the determination of GABA in physiological fluid samples, as described in a recent paper (Song *et al.*, 2005). This new method is about 500 times more sensitive than previously reported HPLC–MS methods and, therefore, well suited to quantify trace levels of GABA present in physiological fluids. A linear calibration curve from 10 to 250 ng/Ml GABA with an $r2$ value of 0.9994 was obtained. Detection limit was estimated to be 5.00 ng/mL GABA (S/N = 3). Human plasma and CSF samples were analyzed. The concentrations of GABA were found to be 98.6 ± 33.9 ng/mL (mean ± S.D., $n = 12$), and 44.3 ± 10.0 ng/mL ($n = 6$) in plasma and CSF, respectively. The method is based on capillary liquid chromatography (LC)/tandem mass spectrometry (MS/MS) using deuterium-labeled GABA (gamma-aminobutyric acid-2,2-D_2, GABA-d_2) as internal standard. Further details of the method can be obtained from the paper.

Elevated plasma GABA may be a marker of autism (Fig. 1). In replication of an earlier study (Dhossche *et al.*, 2002), our research group has found increased plasma GABA levels in a larger sample of prepubertal autistic children (N = 24, ages 3–12). Children with autism had higher plasma GABA levels than control children (without autism or any other neuropsychiatric conditions) (T-test,

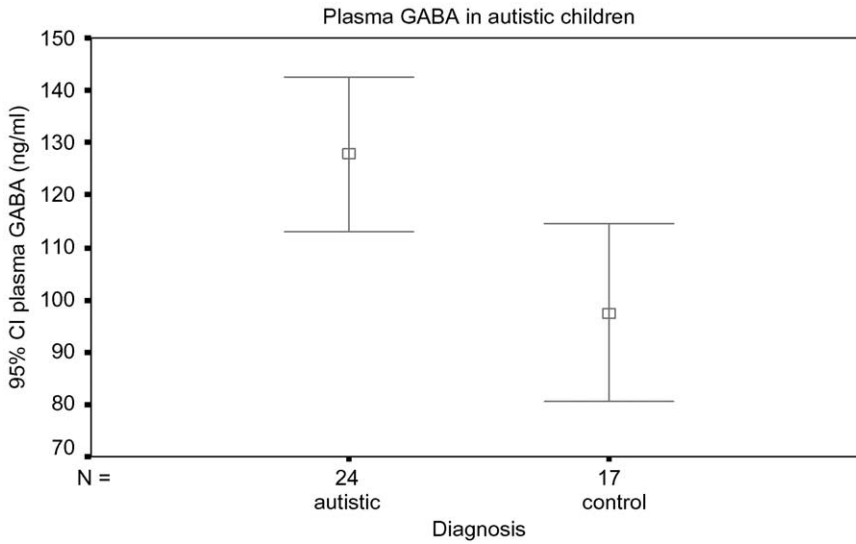

FIG. 1. Plasma GABA levels in autistic children versus controls (p = 0.008).

t = 2.8, df = 39, p = 0.008). Age and sex were not different between diagnostic groups. Although psychotropic medications and certain antiepileptic medication may alter plasma GABA levels (Kemph *et al.*, 1993; Lichtshtein *et al.*, 1978; Löscher and Schmidt, 1981; Shiah *et al.*, 2000), findings were similar when comparing plasma GABA levels in autistic children who were drug-naïve or without psychotropic medications for a least one month versus those who were on various psychotropics.

Low plasma GABA levels may be a novel familial marker of autism (Fig. 2). In a separate study, we measured plasma GABA in 41 parents of children with and without autism (see box plot of parental plasma GABA levels; median, range, the box length represents the middle 50% of the data). Median GABA levels were 63.7 ng/ml for mothers of autistic children (N = 12), 75.5 ng/ml for fathers of autistic children (N = 7), 120.5 ng/ml for mothers of control children (N = 14) (without autism or any other psychiatric disorder), and 99.6 ng/ml for fathers of control children (N = 8). Plasma GABA levels were significantly lower in parents of autistic children compared to control parents (81.3 ng/ml SD 32.2 versus 112.2 ng/ml SD 38.5; Mann-Whitney test, Z = −2.6, p = 0.008). Plasma GABA levels were significantly different between mothers of autistic children versus control mothers (Mann-Whitney test, Z = −2.4, p = 0.02), but not between fathers of autistic children and control fathers (Mann-Whitney test, Z =−1.1, p = −0.28).

Age was not different between the two groups of fathers and mothers. Information on the menstrual phase was not collected although there is one study showing increased plasma GABA levels from the follicular to the luteal

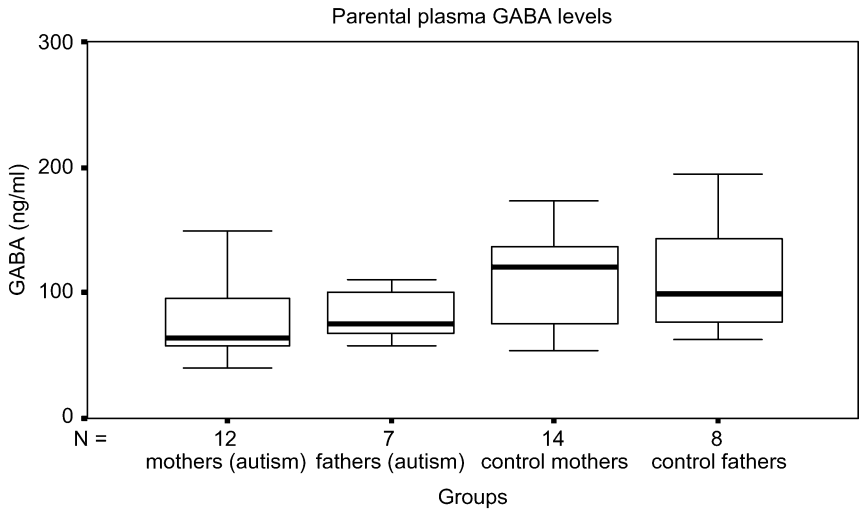

FIG. 2. Plasma GABA levels in parents of children with and without autism (p = 0.008).

phase (Halbreich *et al.*, 1996). None of the mothers indicated pregnancy. In one study, CSF GABA was lower during pregnancy (Altemus *et al.*, 2004). More than half of parents (23 of 41, 56%) were medication-free. Others took a wide variety of medications. Psychotropic medications (including antidepressants, psychostimulants, and antipsychotics) were reported in six parents. About the same proportions of mothers of autistic children and control mothers reported taking medications. The literature on changes of plasma GABA due to medications is limited. A definite increase in plasma GABA has only been shown after treatment with divalproic sodium (Löscher and Schmidt, 1981; Shiah *et al.*, 2000). None of the parents was prescribed divalproic acid or any other anticonvulsant. Medications are therefore unlikely to have changed plasma GABA levels in this pilot study. Overall, plasma GABA levels were lower in parents of autistic children than in control parents. The difference was not statistically significant in fathers possibly owing to the small sample size. Replication in a larger study is warranted.

There are no studies on the familial aggregation of plasma GABA levels in relatives of autistic probands. Twin and family studies have shown that plasma GABA levels are to some extent under genetic control (Petty *et al.*, 1999). In a family study (Bjork *et al.*, 2001), low plasma GABA levels were correlated with measures of aggression in relatives of probands with unipolar depressive illness. These findings support further inquiry in the familial pattern of plasma GABA level as a marker of genetic risk for neuropsychiatric disorders including autism. Previous studies have found low plasma GABA in people with mood disorders and bipolar disorder. Similar findings in parents of autistic children support the theory of DeLong (2004) that there are shared risk factors between types of autism and mood disorder.

There is a discrepancy in our findings between the higher levels of plasma in autistic probands compared to controls, and the lower plasma GABA levels in parents of autistic probands compared to control parents. Current data do not provide an easy explanation, but differences of age and diagnosis between probands and parents are obvious and should be considered. Based on the pilot data in autistic children and their parents, it is hypothesized that plasma GABA levels capture some aspect of central GABA function that is relevant to the pathophysiology and familial risk of autism. These preliminary data suggest that plasma GABA is a promising marker for autism and familial risk of autism.

IV. Insurgent Catatonia

In the past, catatonia was considered a diffuse syndrome, usually associated with schizophrenia. Some have claimed the disappearance of the syndrome due to obscure reasons (Mahendra, 1981). However, empirical findings highlight its

increased incidence in psychosis and validity as a syndrome and genetically determined phenotype (Beckmann *et al.*, 1996; Stöber *et al.*, 2000). For example, in recent samples of acute psychiatric inpatients, the prevalence of catatonia was between 7–17% (Fink and Taylor, 2003). Most catatonic patients were diagnosed with mood disorders. Many authors link catatonia to manic-depressive illness (Abrams and Taylor, 1976; Bush *et al.*, 1996b). Twenty-five percent or more of manic patients have enough catatonic features to meet the DSM criteria. More than half of catatonic patients have manic-depressive illness. About 10–15% of patients with catatonia meet the criteria for schizophrenia.

Catatonia is not classified separately in DSM-IV but serves as a specifier of schizophrenia, mood disorder, and psychosis not otherwise specified, *but not autism* (American Psychiatric Association, 1994). There is increasing support for catatonia to be considered as a distinct syndrome in its own right (Taylor and Fink, 2003), reminiscent of the original conceptualization of catatonia by Kahlbaum (1874) and later by Leonhard (1979). Although there are no controlled treatment studies in catatonia satisfying current standards for evaluating therapies, the literature is consistent showing positive effects of anticonvulsant drugs, particularly benzodiazepines and barbiturates, and of electroconvulsive therapy (ECT), regardless of the severity or etiology of catatonia (Caroff *et al.*, 2004; Fricchione *et al.*, 1983; Rohland *et al.*, 1993).

In a recent study (Ungavri *et al.*, 2005), using a narrow definition of catatonia (i.e., the presence of four or more signs/symptoms with at least one having a score 2 or above on the Bush–Francis Catatonia Rating Scale (BFCRS)) (Bush *et al.*, 1996b), 72 subjects (32%) with chronic schizophrenia met the criteria for the catatonia group (mean number of catatonic signs/symptoms = 5.9F2.0; mean sum score of 8.7F3.4 on the BFCRS). The frequency distribution of catatonic signs/symptoms in the catatonic group and in the whole sample was very similar, with mannerisms, grimacing, stereotypes, posturing, and mutism being the most frequent. Catalepsy, mannerisms, posturing, and mutism are the features traditionally associated with catatonic schizophrenia.

In another study (van der Heijden *et al.*, 2005), a large sample of schizophrenics (N = 19,309) was studied. Although the diagnosis of catatonic schizophrenia dropped from 7.8% in 1980–1989 to 1.3% in 1990–2001, a possible underdiagnosis of catatonic schizophrenia was found in an independent sample. In a consecutive sample of patients admitted with psychosis, application of a systematic catatonia rating scale showed that 18% fulfilled criteria for catatonia (van der Heijden *et al.*, 2005).

Together, these studies (Ungavri *et al.*, 2005; van der Heijden *et al.*, 2005) suggest under-diagnosis and non-recognition of catatonia both in patients with schizophrenia and bipolar disorder. The reasons are complex, but may include the historical decision to classify catatonia as a type of schizophrenia, the segregation of severe psychiatrically ill patients in long-term facilities, and the

perceived lack of anti-catatonic treatments (Fink and Taylor, 2003). These factors may have suppressed interest in and recognition of catatonia. A study of Wing and Shah (2000) showing that 17% of adolescents and young adults with autism satisfied criteria of catatonia suggests that catatonia may also be underdiagnosed in autism owing to similar or other reasons. This study will be discussed at length in one of the next sections.

V. GABA in Catatonia

> The higher nervous arrangements evolved out of the lower to keep down those lower, just as a government evolved out of a nation as well as directs that nation. If this be the process of evolution, then the reverse process of dissolution is not only "a taking off" of the higher, but is at the same time a "letting go" of the lower. If the governing body of this country were destroyed suddenly, we should have two causes for lamentation: (1) the loss of service of eminent men; and (2) the anarchy of the now uncontrolled people. The loss of the governing body answers to the dissolution in our patient (the exhaustion of the higher two layers of his highest centres); the anarchy anwers to the no longer controlled activity of the next lower level of evolution (third layer).
>
> John Hughlings Jackson (1835–1911)

> Progressive inhibition of tonically inhibited living systems, from cell to society, is coupled with increased variability generation in such a manner that the probability of making an optimally adaptive choice of behavior from among the options available remains constant over a wide range of increasing force parameters.
>
> Eugene Roberts (2000)

The single most important observation leading to the belief that GABA dysfunction plays a role in catatonia is the often dramatic response to treatment with benzodiazepines (i.e., positive modulators of the benzodiazepine/$GABA_A$ receptor complex) (Bush *et al.*, 1996a; Fricchione *et al.*, 1983). Other effective treatments for catatonia (e.g., barbiturates, zolpidem, carbamazepine, and electroconvulsive therapy (ECT)) (Green, 1986; Sanacora *et al.*, 2003), also seem to enhance GABA function. Efficacy of serotonergic agents and antipsychotics in catatonia has been less well documented, but seems less consistent.

If one assumes a central role of GABA dysfunction in catatonia, the scope of GABA functions in the normal brain should allow the expression of catatonia when deficiencies in GABA function develop. Roberts (2000) sees a central role of GABA as neurotransmitter used by neurons that exert tonic inhibition of neural circuits for innate or learned behavioral sequences. In its extreme forms, catatonia is characterized by immobility alternating with purposeless agitation. Both behaviors can be viewed as opposite primitive reflexes in response to overwhelming stress or danger, that are expressed when innate, genetically preprogrammed neuronal circuits are released from tonic inhibition. Following John Hughlings

Jackson's hierarchical concept of dissolution (Jackson, 1958), immobility or hyperactivity are then "positive" symptoms caused by the removal of the influence of higher centers. The neuronal circuitry that is involved in catatonia is not well defined, but probably involves frontal cortex, parietal cortex, basal ganglia, and possibly the cerebellum (Fink and Taylor, 2003).

A few general criteria for any viable GABA theory of catatonia are proposed:

1. The theory has to accommodate findings from GABAergic theories of schizophrenia and affective disorders as catatonia occurs in both disorders.
2. Hypothalamic abnormalities of GABA function should be present and may account for severe autonomic dysfunction in malignant catatonia.
3. Treatments that relieve catatonia should enhance GABA function, directly or indirectly.
4. Genetic studies in catatonia, schizophrenia, and affective disorders should provide support for involvement for genes affecting GABA function, at least in subgroups.

Evidence that GABA dysfunction in catatonia satisfies these criteria is summarized:

1. GABA theories have been formulated for schizophrenia (Roberts, 1972; van Kammen, 1977), psychosis (Kalkman and Loetscher, 2003; Keverne, 1999), and affective disorders (Brambilla *et al.*, 2003; Emrich *et al.*, 1980; Petty, 1995) including bipolar disorder (Petty *et al.*, 1993).

2. GABA is a prominent neurotransmitter in the hypothalamus (Decavel and van den Pol, 1990). Hypothalamic GABAergic mechanisms are considered important for regulation of stress responses by the hypothalamic-pituitary-adrenal (HPA) (Herman and Cullinan, 1997; Engelmann 2004).

3. Most currently used psychotropic medications, including benzodiazepines, antipsychotics (Zink *et al.*, 2004), selective serotonin reuptake inhibitors (Bhagwagar *et al.*, 2004; Sanacora *et al.*, 2002; Tunnicliff *et al.*, 1999), phenelzine (Baker *et al.*, 1991; McManus *et al.*, 1992), and anticonvulsants seem to enhance GABA neurotransmission, albeit through different mechanisms. A few studies suggest a direct role of GABA in ECT. In an iomazenil-SPECT study, increased benzodiazepine receptor uptake in cortical regions (except temporal cortices) was found one week after successful bitemporal ECT (Mervaala *et al.*, 2001). In a MRS study, two-fold increased brain GABA levels were found in depressed patients after a course of ECT (Sanacora *et al.*, 2003). The small number of patients precluded conclusions regarding any correlation between clinical improvement and increased brain GABA level. Previously, it was reported that CSF GABA increased by 50% after ECT (Lipcsey *et al.*, 1986). In another study, cortical glutamate/glutamine levels in the left anterior cingulum of depressed patients normalized after ECT, but only in responders (Pfleiderer *et al.*, 2003). In

non-responders, levels remained low. Limitations in this study's MRS methodology did not allow obtaining separate measurements for GABA because of overlapping resonances of glutamate, glutamine, and GABA. In a study of plasma GABA in depressed patients treated with ECT, plasma GABA levels tended to decrease for about one hour after ECT (Devenand *et al.*, 1995). Limitations of this study include variable storage times known to increase GABA levels in plasma and CSF and PRN administration of chloral hydrate that enhances GABA function similar as barbiturates, during the ECT course.

4. There is some evidence that GABA related genes are involved in affective disorder and schizophrenia. GABRA5 has been associated with bipolar disorder in two studies (Otani *et al.*, 2005; Papadimitriou *et al.*, 1998). In a genome scan of catatonia, a linkage signal in the region 15q11.2-q21.1 (where three GABA-A subunit genes are located) was found (Stöber *et al.*, 2000). There are no (family-based) gene association studies in catatonia available in the literature. Findings from a family-based association study in a sample of children and adolescents with childhood-onset schizophrenia (COS) (n = 72) suggested that the gene encoding GAD(67) may be a common risk factor for schizophrenia (Addington *et al.*, 2005).

Empirical evidence for GABA dysfunction in catatonia comes from one single receptor-imaging study. Findings in a benzodiazepine ligand-binding study of catatonic patients have shown a decreased density of $GABA_A$ receptors in the left sensorimotor cortex in akinetic catatonia (Northoff *et al.*, 1999). Other, more circumstantial, evidence is found in biochemical studies. Cerebrospinal fluid levels of GABA were decreased in 11 patients with Neuroleptic Malignant Syndrome, a condition that may be related to catatonia, compared with GABA CSF levels in eight controls (Nisijima and Ishiguro, 1995). In this study, levels of noradrenalin were increased, but levels of 5-hydroxyindoleacetic acid (5-HIAA), serotonin's main metabolite, were slightly, but not significantly, decreased. Further evidence of impaired GABA function in catatonia must await future studies.

VI. Catatonia in Autism

A. Case-Vignette (Dhossche, 1998)

John was the full-term product of an uneventful pregnancy. Birth was complicated by cord strangulation and hypoxia. He was described as a peaceful baby. At age 2, John did not speak any words yet, he was overly quiet and passive. Hearing deficits were suspected by his mother. Audiological testing indicated normal hearing.

At age 3, he was evaluated by a child neurologist and child psychiatrist because of problems with speech development and poor social interactions. Psychiatric observation showed avoidant gaze, limited interest in social interactions, limited emphatic contact, inappropriate smiling, and bizarre postures. His vocabulary was limited to a few words. He also used many jargon words and was echolalic. John was greatly fascinated by flickering lights and twirling objects. Neurological investigations were negative, including urinary amino acids, CSF, and pneumoencephalograph. The EEG was abnormal, with mild general slowing. A diagnosis of Autistic Disorder was made. John was placed in special education. His adjustment at school and home improved over time. Social functioning remained poor. Insistence on routines and obsessive preoccupations were constantly present throughout this period. Developmental progress was somewhat jerky. At age 5, his speech improved greatly in a short period. He also showed more interests. Cognitive testing was done at age 11 and showed a total IQ (WISC-R) of 103.

When John was 15, he started to complain about command auditory hallucinations. There was also marked worsening of obsessive-compulsive behaviors, including obsessive slowness. His psychomotor retardation alternated with outbursts of aggression and agitation. School performance deteriorated gradually. He was admitted to the psychiatric hospital.

Initial psychiatric assessment showed a disheveled adolescent with alternating episodes of agitation and severe psychomotor slowing, staring in space, waxy flexibility, posturing, and decreased verbal output. He reported command hallucinations. There was no formal thought disorder. Obsessions were elaborate and had a delusional quality. His mood was constricted. No focal neurological abnormalities were found. CT of the brain and routine laboratory tests were normal. Chromosomal analysis showed 46 XY karyotype without fragile X. Schizophrenia with catatonia and paranoid features, superimposed on AD, was diagnosed.

The patient was treated with several antipsychotic medications but his condition did not improve. John continued to complain about hearing voices. He was aggressive and difficult to manage on the ward. Episodes of catatonic behavior were among the most prominent features throughout the first year of admission. At that point, clozapine was prescribed in monotherapy (400 mg per day). Improvement was seen after a few weeks. John became less aggressive and more social. Catatonic recurrences continued. The frequency of stuporous episodes decreased after lorazepam (2.5 mg three times a day) was added to this medication regimen. A few months later, John was discharged from the hospital. Follow-up shows a young man in his early twenties with autistic symptoms but without catatonic-psychotic recurrences. He attends a day treatment center and lives with his parents. He continues to take clozapine and a small dose of lorazepam (2.5 mg/day).

B. Comments

The clinical presentation of this patient carries some similarity with Neuroleptic Malignant Syndrome (NMS). NMS is an unlikely diagnosis because the onset was before antipsychotic medications were prescribed. During the protracted inpatient course the catatonic syndrome seemed refractory to treatment with various typical antipsychotics. It is possible that catatonia was sustained or exacerbated by this medication regimen.

The marked response to clozapine may indicate superior effect on catatonia (Battegay *et al.*, 1977). On the other hand, there is anecdotal evidence that clozapine improves behavioral problems in autistic children (Zuddas *et al.*, 1996). The positive response to lorazepam suggests specificity of benzodiazepine treatment in catatonic stupor, in accord with controlled studies in general psychiatric patients (Bush *et al.*, 1996a; Ungvari *et al.*, 1994).

The case of John is typical for AD until the onset of psychosis. Hallucinations and delusions in AD are considered rare but have been reported, mostly in high-functioning patients (Kurita, 1999; Petty *et al.*, 1984). Obsessions were difficult to differentiate from delusions. Auditory hallucinations were prominent suggesting a diagnosis of schizophrenia. However, the catatonic symptoms were equally disabling. The progression of stereotypical movements, compulsions, withdrawal into full catatonia is remarkable and has been observed by others (Wing and Shah, 2000; Zaw *et al.*, 1999). In that respect, autistic symptomatology appears as a *form fruste* of full catatonia.

Other case-reports show that catatonia emerges in some people with autism (Ghaziuddin *et al.*, 2005; Realmuto and August, 1991). There seems to be considerable overlap of psychomotor symptoms between the two disorders (e.g., muteness, echolalia, stereotypical movements, and other psychomotor peculiarities). There is one systematic study (Wing and Shah, 2000) of catatonia in autism showing that 17% of a referred sample of adolescents and young adults with autism satisfied modern criteria for catatonia. A semi-structured interview was used to collect information from parents or other caregivers. Patients were diagnosed with catatonia when an exacerbation of certain behavioral features occurred in sufficient degree to interfere with everyday functions of self-care, education, occupation, and leisure. Essential features of catatonia were: increased slowness affecting movements and verbal responses, difficulty in initiating and completing actions, increased reliance on physical or verbal prompting by others, and increased passivity and apparent lack of motivation. Other associated characteristics were reversal of day and night, Parkinsonian features (tremor, eye-rolling, dystonia, odd stiff posture, freezing in postures), excitement and agitation, and increase in repetitive, ritualistic behavior.

Thirty individuals with autism aged 15 or older met criteria for catatonia. Classic autism (AD) was diagnosed in 11 (37%), atypical autism (PDD NOS) in 5

(17%), and Asperger Disorder (AsD) in 14 (47%). All of those with catatonia were aged 15 or older. None of those under age 15 had the full syndrome although isolated catatonic symptoms were often observed. In the majority of cases, catatonic symptoms started between 10 and 19 years of age. Five individuals had brief episodes of slowness and freezing during childhood, before age 10. Obsessive-compulsive and aggressive behavior preceded catatonia in some. Visual hallucinations or paranoid ideas were occasionally reported, but no diagnosis of schizophrenia could be made. Referred patients with catatonia were significantly more likely than patients without catatonia to have had impaired language and passivity in social interaction before the onset of catatonia. Family history and treatments were not recorded.

This study is the only published systematic assessment of catatonic symptoms in autistic people. Although the authors did not use DSM-IV criteria of catatonia, their definition of catatonia includes several DSM-IV core symptoms including severe psychomotor retardation, decreased verbalizations, posturing, and agitated episodes. The high prevalence of catatonic symptoms in autism suggests an intricate, but unaccounted, relation between autistic and catatonic symptoms.

VII. Catatonia in Prader-Willi Syndrome

> During lethargic-refusal states, the patient [with Prader-Willi Syndrome] is practically bedridden, refuses any human approach, food or drink, and sinks into a state of self neglect leading to soiling with no evidence of pathophysiologically determined incontinence. These lethargic-refusal states can last for weeks or months, and may resolve spontaneously or with the help of behavioral or pharmacological interventions.
>
> G. Bartolucci and T. Younger (1994)

A. Case-Vignette (Dhossche and Bouman, 1997)

Mark was the only child of well-educated parents. He was born after 40 weeks of normal gestation. Birth weight was 2461 grams. Apgar score was 7 (after 1′), and nine (after 2′). A pediatric referral was made at 2 months because of hypotonia, feeding problems, hypogonadism, and bilateral retentio testis. EEG, brain CT, and amino acid analysis were normal. Chromosome analysis showed 46 XY configuration. Prader-Willi syndrome (PWS) was diagnosed on clinical grounds. No further cytogenetic studies were done.

Mark started walking when he was 18 months old. He spoke his first words at 14 months and whole sentences at age 3. During the next few years, stigmata of

PWS (e.g., excessive appetite, obesity, short stature, oppositional behavior, and temper tantrums) became clear. Cognitive testing showed scores in the mildly retarded range (IQ 60–65 on WISC-R). He was cheerful as a child. There was no personal or family history of psychosis, epilepsy, or autism.

Mark lived with his parents in a stable family environment. He was well adjusted and he attended a special education school for mentally retarded youngsters. Occasional temper tantrums responded well to behavioral interventions. A few months before the onset of illness, the family moved and Mark started attending a new school, without any obvious adjustment problems.

At age 17, Mark was admitted to a pediatric hospital because of acute onset of catatonia with stupor, staring, incontinence, mutism, rigidity, waxy flexibility, posturing, refusal to eat and drink, and severe disruption of the sleep-wake cycle. Full-blown catatonia developed within one day. A few hours before the onset, Mark had a rare but vehement argument with his mother regarding some school matter. He was sent to his room. When he came back, he was very anxious, talked incoherently about Jesus, and made vague references about visual hallucinations. Within hours, he became catatonic and was brought to the hospital.

Initial treatment consisted of supportive medical care and haloperidol (1 mg three times daily). His condition remained unchanged. He lay in bed, mute, and withdrawn. Neurological examination showed clear consciousness, normal reflexes, intact cranial nerves, drooling, and increased muscle tone. No paresis or sensory abnormalities were found. EEG showed mild, nonspecific slowing in the prefrontal areas. Red and white blood cell count, serum electrolytes, and liver function tests were all within normal limits. The consulting neurologist failed to find evidence for a neurological disorder, and concurred with the diagnosis of stupor of psychiatric origin. He thought that drooling and increased muscle tone were caused by haloperidol.

After 10 days of minimal improvement, a test dose of lorazepam (1 mg) was given orally. Considerable improvement of catatonia occurred within hours. The next day, Mark started walking and eating again. He became more verbal and answered simple questions adequately. He also started making delusional statements that his parents had died, and reported visual hallucinations of his grandparents and Santa Claus. Lorazepam was increased to 4 mg daily over the course of a few days. Stupor gave way to motor restlessness, short attention span, impulsiveness, stereotypies, echolalia, echopraxia, automatic obedience, active resistance to movement, ambitendency, and negativism. Mark continued to make nihilistic-delusional statements for which risperidone was started, in addition to lorazepam, and increased to 6 mg daily. Catatonia and delusions resolved during the next two weeks. Mark was discharged from the hospital and returned back to baseline functioning. Risperidone and lorazepam were discontinued 2 months after discharge. No relapses occurred during five-years follow-up.

B. Comments

PWS is a genetic disorder characterized by hypotonia at birth, small hands and feet, almond shaped eyes, hypogonadism, short stature, and diabetes. Most patients exhibit mild to moderate mental retardation and obesity. Obesity starts in infancy and is accompanied by compulsive eating. The prevalence is estimated at one in 16,000–25,000 live births (Burd *et al.*, 1990; Smith *et al.*, 2003).

This multi-system disorder occurs in all races and both sexes and arises from the lack of expression of genes on the paternally derived chromosome 15q11–13. Candidate genes for PWS in this region are imprinted and silenced on the maternally inherited chromosome. The genetic defect underlying PWS is the absence of expression of one or more genes of paternal origin located on the long arm of chromosome 15 (15q11-13). Several genetic mechanisms have been associated with PWS, mainly paternal 4Mb deletion (in about 60% of cases) and maternal uniparental disomy (UPD) (in about 25% of cases) (Woodage *et al.*, 1994). In a small number of patients (3%), imprinting errors are found because of either a sporadic or inherited microdeletion in the imprinting center. There is a paternal chromosomal translocation in 1% of the cases. Imprinting occurs partly through parent-off-origin allele-specific methylation of CpG residues which is established either during or after gametogenesis and maintained throughout embryogenesis. Alternatively, if the deletion is maternal or there are two paternal copies of chromosome 15q, another syndrome is found (e.g., Angelman Syndrome (AS) characterized by severe mental retardation, attention deficit, inappropriate laughter, ataxia, jerky gait, epilepsy, sleep disturbances, and craniofacial abnormalities) (Angelman, 1965).

Mark satisfied clinical criteria of PWS (Åkefeldt *et al.*, 1991; Holm *et al.*, 1993). Severe neonatal hypotonia, hypogonadism/delayed sexual maturation, obesity, and learning problems are major criteria. All major features were present in Mark. This established the diagnosis with high probability. Unfortunately, no cytogenetic or molecular testing was done.

Psychosis and catatonia started suddenly. There were no prodromal symptoms or premorbid signs of any psychiatric disorder. The episode lasted less than 1 month and there was full return to premorbid level of functioning. These features suggest a diagnosis of Brief Psychotic Disorder, according to DSM-IV (American Psychiatric Association, 1994). The case also satisfies criteria for the DSM-IV catatonic specifier. A clear precipitant of the episode was present, satisfying the specifier "with marked stressors." There was a severe quarrel between Mark and his mother hours before his psychotic break. Possible contributing factors were the recent move and change of school. Although the subsequent course seems out of proportion to the original precipitants, these

events may well qualify as very stressful for Mark considering his restricted emotional and cognitive coping.

Catatonic stupor responded quickly to treatment with lorazepam, in accordance with studies in adults (Bush *et al.*, 1996a; Fricchione *et al.*, 1983; Ungvari *et al.*, 1994). A different set of catatonic symptoms appeared after resolution of stupor with lorazepam. It was clear that Mark was delusional and hallucinating once he became verbal again. He responded well to the addition of risperidone. Others have reported positive effects of atypical antipsychotics, including risperidone, in adult catatonia without developmental disorders (Battegay *et al.*, 1977; Cook *et al.*, 1996). More studies are needed to assess the relative and differential efficacy of benzodiazepine monotherapy versus combined regimens (benzodiazepine and atypical antipsychotic) in the treatment of catatonia.

Studies have shown that compulsions (Holm *et al.*, 1993; Wigren and Hansen, 2003) and psychotic disorders (Boer *et al.*, 2002; Clarke, 1993) are associated with PWS. Compulsive behaviors in PWS are autistic-like and include insistence of sameness (Wigren and Hansen, 2005), perseveration, and ordering. Hand washing and checking (i.e., symptoms that are typically found in Obsessive-Compulsive Disorder) are infrequent in PWS. Various psychotic symptoms including paranoia, hallucinations, and bizarre behavior, have been reported in PWS patients, usually in combination with affective symptoms (Clarke, 1993; Whitman and Accardo, 1987). The acute onset and mixture of affective and psychotic symptoms suggest an atypical form of psychosis.

PWS patients with uniparental disomy seem particularly prone to develop psychosis (Boer *et al.*, 2002; Verhoeven *et al.*, 1998; Vogels *et al.*, 2003). This suggests that an abnormal pattern of expression of sex-specific imprinted genes on 15q11–13 is a risk factor for psychosis in PWS. Superior visual recognition memory is another feature in PWS that has been associated with maternal uniparental disomy (Joseph *et al.*, 2001). The genes involved in the development of pychosis and superior recognition memory in PWS are unknown.

The case-vignette is the only case-report in the literature of an adolescent with PWS who developed full-blown catatonia (Dhossche and Bouman, 1997). Catatonic symptoms have been described in a few other cases, but have not been rigorously assessed. For example, Clarke (1993) reported an acutely psychotic PWS patient with significant psychomotor retardation, refusal of food and fluids, and negativistic behavior. Abe and Ohta (1995) described an adolescent with PWS who developed stupor, pallor, and delusional thinking. Bartolucci and Younger (1994) observed, in four of nine youngsters with PWS, discrete episodes of a refusal-lethargy syndrome, characterized by akinesis, refusal of foods and fluids, incontinence, and self-neglect, that occurred independently from psychotic episodes and lasted weeks up to several months. These descriptions suggest the presence of catatonia. Catatonia might have gone unrecognized, as some

catatonic symptoms are transient and need to be elicited by trained clinicians. Systematic studies are needed to assess the prevalence of catatonia in PWS.

VIII. GABA in Prader-Willi Syndrome

A few studies have suggested abnormal GABA metabolism in PWS. In one study (Ebert *et al.*, 1997), plasma GABA levels were two to three times higher in people with PWS (and Angelman Syndrome) than controls. This finding was not explained by obesity or level of cognitive impairment. Within the group of subjects with PWS, genetic status (chromosome 15 deletion or disomy) was not related to higher plasma GABA levels. Cerebral $GABA_A$ receptors were studied in PWS (Lucignani *et al.*, 2004). A reduction of [11-C]flumazenil (a ligand of the benzodiazepine binding site associated with the $GABA_A$ receptor) was found in several cortical brain areas in adults with PWS compared to controls. Findings of abnormal GABA metabolism and $GABA_A$ receptors may be related to abnormal expression of $GABA_A$ receptor subunit genes (GABRB3, GABRA5, and GABRG3) located on the PWS/AS chromosomal region (15q11–13) (Lalande *et al.*, 1994; Wagstaff *et al.*, 1991). Some indirect evidence comes from studies showing that treatment with topiramate, an anticonvulsant with GABA-ergic effects, reduces stereotyped and compulsive behaviors in PWS patients (Shapira *et al.*, 2002, 2004; Smathers *et al.*, 2003).

Hypothalamic dysfunction is considered one of the key features of PWS (Swaab, 1997). Abnormal hypothalamic function in PWS may be associated with excessive eating, lack of satiation, growth hormone deficiency, hypogonadism, daytime hypersomnolence, and abnormalities in body temperature control. There are very few neuropathological studies of the hypothalamus in PWS. Swaab *et al.* (1995) found smaller paraventricular nuclei (28% reduction), fewer oxytocin-expressing cells (54% reduction), but normal numbers of vasopressin-expressing cells in the hypothalamus of 5 individuals with PWS. To our knowledge, there are no studies in the literature providing direct evidence of abnormal GABA function in the hypothalamus of individuals with PWS. Given the importance of GABA as neurotransmitter in the hypothalamus (Decavel and van den Pol, 1990), such studies are warranted.

PWS and AS are contiguous gene syndromes (Schmickel, 1986). This type of genomic disorder is characterized by large chromosomal rearrangements with complex phenotypes associated with the dosage effects of multiple unrelated genes. The region on chromosome 15q11-q13 is an example where multiple structural abnormalities, including deletions, duplications, triplications, and supernumerary marker chromosomes have been reported, especially in cases with

autism (Lauritsen *et al.*, 1999). Abnormalities of the proximal long arm of chromosome 15 have emerged as the most frequent (up to 3%) cytogenetic abnormality in the autistic population (Bolton *et al.*, 2001; Schroer *et al.*, 1998). Detection is done by testing through FISH studies and additional molecular analyses to determine parental origin of the duplications or abnormal gene segment. So far, all duplications in this region have been maternal in origin (Cook *et al.*, 1997). This suggests that an imprinted gene may be involved in those cases. In cases with inverted duplicated chromosome 15, autistic symptoms are present in varying degrees of severity (Borgatti *et al.*, 2001a). Plasma GABA levels were higher, but not significantly, in cases with this syndrome compared to controls (Borgatti *et al.*, 2001b). The negative finding may be due to the small sample size ($N = 8$) of the study. This suggests that the GABAergic system may be implicated in patients with inverted duplicated chromosome 15 as well as in some patients with idiopathic autism.

Altered brain $GABA_A$ receptor function may account for some of the neuropsychiatric problems associated with PWS as there is increasing evidence that GABA neuronal dysfunction is implicated in various psychiatric disorders including psychosis (Guidotti *et al.*, 2000; van Kammen *et al.*, 1998), mood disorders (Sanacora *et al.*, 1999), anxiety disorders (Goddard *et al.*, 2001), and autism (Blatt *et al.*, 2001; Dhossche *et al.*, 2002). Genetic studies also suggest that polymorphisms of a $GABA_A$ receptor subunit gene (GABRB3) or a gene near this locus increases susceptibility to catatonia (Stöber *et al.*, 2000, 2002), bipolar disorder (Papadimitriou *et al.*, 1998; Otani *et al.*, 2005), and autism (Nurmi *et al.*, 2003; Shao *et al.*, 2003). GABRB3 has been associated with a subtype of autism with high levels of repetitive behaviors and stereotyped patterns (insistence on sameness) (Shao *et al.*, 2003) and with a subtype of autism with savant skills, a characteristic often associated with the development of special skills (O'Conner and Hermelin, 1991). Repetitive or obsessive-compulsive behaviors (Clarke *et al.*, 2002; Dykens *et al.*, 1996), splinter skills (Dykens, 2002), and catatonia (Dhossche and Bouman, 1997) have been associated with PWS. Taken together, these studies suggest that a genetic locus (or loci) in the PWS region (possibly GABRB3 or nearby genes) confer(s) risk for repetitive, ritualistic, and obsessive-compulsive behaviors. Future studies of the association between these psychopathological dimensions and GABAergic parameters are warranted.

IX. Conclusion

There are supportive data for GABA dysfunction in autism, Prader-Willi syndrome, and catatonia. Modern in vivo techniques to measure GABA in the plasma and brain allow further clinical studies to examine common GABAergic

mechanisms in these disorders. Such studies may ultimately lead to improved treatments for selected symptoms in these disorders.

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ALCOHOL, GABA RECEPTORS, AND NEURODEVELOPMENTAL DISORDERS

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Development of the fetal brain is affected by maternal alcohol consumption during pregnancy. Children exposed to alcohol during gestation suffer from a wide range of physical and neurological damage. Both heavy and moderate maternal drinking is unsafe for the developing fetus. Heavy drinking causes obvious physical and behavioral changes in children, whereas moderate drinking may cause only subtle changes that may remain unnoticed. Neurobehavioral problems in children resulting from both heavy and moderate maternal drinking during pregnancy are long-lasting and are aggravated by stress and the natural process of aging. There are several ways alcohol can disturb the normal process of brain development. These include changes in neurotransmitters, their receptors, and the process of neurotransmission. Recent studies provide strong evidence that γ-aminobutyric acid (GABA) and GABA-receptor systems are affected by alcohol during brain development. GABA and GABA-receptors play significant roles in the proliferation, migration, differentiation, and positioning of different cell types in the developing brain required for the optimum synaptogenesis and balance in excitatory and inhibitory neurotransmission. Studies with rodent brains clearly indicate that alcohol during pregnancy alters these processes by GABA and GABA-receptor dependent mechanisms. This chapter provides an overview on the GABA-receptor system in rodent brains, the role of this system in the normal development of the brain, and disturbances that may cause neurobehavioral disorders with an emphasis on alcohol.

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I. Introduction

Maternal alcohol consumption during pregnancy is one of the leading causes of neurobehavioral problems in the Western world. Children exposed to alcohol during gestation suffer from a wide range of physical and neurological abnormalities commonly known as fetal alcohol syndrome (FAS) (Jones *et al.*, 1973; Ulleland, 1972). The characteristics of FAS include growth deficiency, short palpebral fissures, a relatively short nose, indistinct philtrum, thin upper lip, flattened medial midface, small crania, cognitive impairment, intellectual deficiencies, behavioral disturbances, and neurological damage (Sampson *et al.*, 1997).

Studies with human brains that are exposed to alcohol during gestation show that not all parts of the brain are equally sensitive to alcohol's teratogenic effects. Autopsy and magnetic resonance studies combined with quantitative analysis reveal that neuronal migration, basal ganglia (both caudate and lenticular nuclei), cerebellum (anterior vermis; lobule I to V), and corpus callosum (complete to partial agenesis, hypoplasia, and midline abnormalities) are primarily vulnerable to heavy alcohol exposure (Bhatara *et al.*, 2002; Johnson *et al.*, 1996; Jones *et al.*, 1974; Mattson *et al.*, 1992, 1996, 2001; Riley *et al.*, 1995; Swayze *et al.*, 1997). Ocular and auditory abnormalities are also common amongst children with FAS (Church and Kaltenbach, 1997; Stromland and Pinazo-Duran, 1994). Positron emission tomography analyses seem to show low levels of glucose metabolism in the caudate and cerebellum of children with FAS (Roebuck *et al.*, 1998), suggesting zone specific metabolic deregulation induced by prenatal alcohol in human brains.

Moderate alcohol consumption during pregnancy causes subtle impairments in the behavior of offspring that may occur in absence of characteristic facial and severe behavior abnormalities of FAS. This condition is categorized as alcohol related neurodevelopmental disorder (ARND). Cognitive deficits in children with ARND are often not apparent until the child is challenged during the educational years (Conry, 1990; Streissguth *et al.*, 1990) or later in life (Streissguth *et al.*, 1991). Therefore, behavioral consequences of gestational exposure to moderate alcohol may have long lasting effects (Riley, 1990). Signs may diminish as the victim develops compensatory mechanisms to deal with the dysfunction but re-emerge under stressful conditions or with age (Steinhausen and Spohr, 1998). Higher incidence of adult-onset of psychiatric disturbances, including a 44% incidence of major depressive illness and 40% incidence of psychosis is reported in humans exposed to alcohol during gestation (Famy *et al.*, 1998). Animal studies also demonstrate this nature of the problem (Hannigan *et al.*, 1987; Riley, 1990). The combined rate of FAS and ARND is estimated to be at least 9 out of 1000 live births in developed countries (Sampson *et al.*, 1997). Recent studies also document autism in children exposed to alcohol during gestation (Miles *et al.*, 2003; Nanson, 1992).

This Chapter presents a brief overview on γ-aminobutyric acid (GABA) and GABA-receptor systems with empasis on their expression and regulatory function during brain development. Data from rodent models are used to describe the roles of this system in the development of neurobehavioral problems resulting from gestational exposure to alcohol. When possible, information on human studies is provided, but the article is primarily supported by data from rodent models.

A. GABA and GABA Receptors

GABA is a highly flexible molecule, exists in many low-energy conformations, and binds to different GABA receptors to mediate its actions. In the mature CNS, three classes of GABA receptors are identified (Chebib and Johnston, 1999). Of these, $GABA_A$ and $GABA_C$ types are ionotropic receptors that form ligand-gated Cl^- ion channels (Feigenspan *et al.*, 1993; Sivilotti and Nistri, 1991). The $GABA_B$ type of receptors are metabotropic (G protein-coupled) and are coupled to Ca^{2+} and K^+ channels or adenylate cyclase via $G_{i/o}$ GTP binding proteins (Kerr and Ong, 1992). $GABA_A$ and $GABA_C$ receptors mediate fast neurotransmission, whereas $GABA_B$ receptors mediate slow neurotransmission (Wong *et al.*, 2003).

The $GABA_A$ receptors display extraordinary structural heterogeneity resulting from various subunit compositions. There are eight subunit families, altogether consisting of 21 different subunits (α^{1-6}, β^{1-4}, γ^{1-4}, δ, ε, π, θ, ρ^{1-3}) that may form a hetero-oligomeric $GABA_A$ receptor. However, in the endoplasmic reticulum and Golgi apparatus, a restricted number of subunit combinations assemble together and are properly packaged, processed, and trafficked to the cell surface (Barnes, 2000). Most *in vivo* $GABA_A$ receptors are formed by the co-assembly of α-, β-, and γ-subunits (Macdonald and Olsen, 1994). Hetero-oligomeric $GABA_A$ receptors are blocked by alkaloid bicuculline and are modulated by barbiturates, benzodiazepines, neuroactive steroids, and alcohol (Chebib and Johnston, 1999; Mehta and Ticku, 1999).

$GABA_C$ receptors are made up of a single type of protein subunit (ρ). The molecular subunits of $GABA_C$ receptors are found in the retina, thalamus, pituitary, and gut. Five different ρ subunits are identified, of which $\rho 1-$, $\rho 2-$, or $\rho 3$-subunits form functional homomeric or pseudoheteromeric receptors (Chebib, 2004). $GABA_C$ receptors are not blocked by bicuculline, nor are they modulated by steroids, barbiturates, or benzodiazepines (Johnston, 1996). GABA is an order of magnitude less potent at $GABA_A$ than $GABA_C$ receptor (Chebib and Johnston, 1999). Muscimol and its analogue, 4,5,6,7-tetrahydroisoxazolo [5,4-c]pyridine-3-ol (THIP), act at both $GABA_A$ and $GABA_C$ receptors. However, at $GABA_A$ receptors, muscimol is an antagonist, whereas THIP is a partial agonist (Krogsgaard-Larsen *et al.*, 1994; Kusama *et al.*, 1993). At $GABA_C$

receptors, muscimol is a partial agonist (Kusama *et al.*, 1993), while THIP is an antagonist (Woodward *et al.*, 1993).

The $GABA_B$ receptors consist of two subunits, $GABA_{B1}$ and $GABA_{B2}$. Different spliced variants of $GABA_{B1}$ receptors are reported and designated as $GABA_{B1a}$, $GABA_{B1b}$, $GABA_{B1c}$, $GABA_{B1d}$, $GABA_{B1e}$, $GABA_{B1f}$, and $GABA_{B1g}$ (Billinton *et al.*, 2001; Bowery *et al.*, 2002; Kawakami *et al.*, 2004). The functional $GABA_B$ receptors require co-assembly of $GABA_{B1}$ and $GABA_{B2}$ (Bowery *et al.*, 2002; Couve *et al.*, 2000). $GABA_B$ receptors are hetero-oligomeric receptors made up of a mixture of subunits, and are selectively activated by baclofen and CCGP27492 and are blocked by phaclofen, the phosphoric analog of baclofen (Chebib and Johnston, 1999).

In adult brains, GABA acts as a major inhibitory neurotransmitter. However, it also mediates excitatory effects in the dorsal root ganglion, CA1 hippocampal pyramidal cells, and cells in layer 5 of cortex in mature brains (Stein and Nicoll, 2003).

B. GABA, GABA Receptors, and Brain Development

1. *Expression Pattern*

GABA is in abundance and is widespread during embryonic development. It appears with the beginning of neurogenesis when the central nervous system is primarily composed of proliferating neuroepithelium (Behar *et al.*, 1996). In the latter phase, GABAergic neurons become widespread in all the regions of the brain (Zecevic and Milosevic, 1997). The widespread appearance of GABAergic neurons in the cortex and hippocampus during the primary period of neurogenesis is consistent with the notion that GABA acts as a trophic signal during neurogenesis (Barker *et al.*, 1998; Ma and Barker, 1998). Since synapses are not present during neurogenesis in the developing brain, it is considered that GABA is released from precursor cells and acts in a paracrine manner to activate its receptors (Demarque *et al.*, 2002). GABA expression is mostly reduced during the perinatal period as the astrocytes and oligodendrocytes appear and neurons differentiate into transmitting circuits (Zecevic and Milosevic, 1997).

During embryonic development different $GABA_A$ receptor subunits exhibit regional and temporal changes with expression levels of some subunits being more prominent than others. Expression of α_2, α_3, α_4, α_5, β_1, β_2, β_3, γ_1, γ_2, and γ_3 dominates in the embryonic stage, whereas in the adults, expression of α_1, α_2, α_3, β_2, β_3, γ_2, and δ predominates (Ma and Barker, 1995). Expression levels of α_2, γ_1, and γ_3 drop progressively during development, whereas the expression of α_1 subunit increases (Fritschy *et al.*, 1994). Functional significance of subunit switching and change in the expression levels during embryonic development is

not completely understood. It is possible that α_4, α_5, and γ_3 subunits form $GABA_A$ receptor/chloride ion channels regulating proliferation, migration, and differentiation of neuronal progenitors rather than synaptogenesis, whereas α_1 subunit may add to form channels for fast GABAergic signals at synapses. The ubiquitous expression of $\beta_{2/3}$ during development possibly allows these subunits to associate with the different α subunits forming distinct receptor subtypes (Fritschy *et al.*, 1994).

The expression pattern of $GABA_B$ subunit mRNA isoforms ($GABA_{B1}$ and $GABA_{B2}$) in developing brain are not coordinately regulated although both transcripts are detected in several structures in developing brains, including the hippocampus, cerebral cortex, neurepithelium, cerebellum, diencephalons, hippocampus, and thalamus (Kim *et al.*, 2003; Li *et al.*, 2004; Martin *et al.*, 2004). The expression of $GABA_{B1}$ subunit during gestation days 11 to 12 is followed by the appearance of $GABA_{B2}$ transcripts about 2 days later. Furthermore, expression of $GABA_{B1a}$ dominates during postnatal development whereas $GABA_{B1b}$ dominates in the adult brains (Fritschy *et al.*, 1999). These differences in the temporal expression patterns of $GABA_B$ receptor isoforms indicate that $GABA_B$ receptor subunit genes are under independent regulation during embryonic development, but the functional significance of this phenomenon is not clear, primarily because information on protein level expression of these subunits are still lacking.

2. *Regulation*

GABA is a diffusible factor and is released from pioneer and migrating neurons (Rivera *et al.*, 2004), growth cones (Gordon-Weeks *et al.*, 1984), and glia (Barakat and Bordey, 2002). GABA in extracellular space is maintained due to delayed maturation of GABA transporters, that although present by the end of the gestation, remain ineffective until the perinatal period (Yan *et al.*, 1997). Diffused GABA acts in a paracrine manner during brain development via GABA receptors (Wang *et al.*, 2003).

Effects of GABA on immature and mature neurons differ. GABA augments levels of intracellular Ca^{2+}, c-Fos, and BDNF transcripts in a GABA-receptor and Ca^{2+} channel dependent manner in rat hippocampal neurons during maturation (Berninger *et al.*, 1995). This GABA effect is seen only in immature neurons because $GABA_A$ antagonists do not influence expression of c-fos and BDNF messenger RNA levels in mature neurons (Berninger *et al.*, 1995; Obrietan *et al.*, 2002).

GABA is excitatory in immature neurons, which switch primarily to their inhibitory actions in the early postnatal brains (Ben-Ari *et al.*, 1989; Obata *et al.*, 1978). Recent studies suggest that this occurs due to changes in the expression pattern of cation-chloride cotransporters (CCCs) during postnatal brain development (Ben-Ari, 2002; Lu *et al.*, 1999). The immature neurons primarily express Na^+-K^+-$2Cl^-$ co-transporter (NKCC1) that is driven by sodium and potassium

gradients and raises intracellular Cl^- concentrations. The K^+-Cl^- co-transporter (KCC2), in contrast, is expressed primarily in mature neurons and couples Cl^- transport to the K+ gradient and normally lowers the intracellular Cl^- concentrations. Since $GABA_A$ receptors are permeable to Cl^-, activation of these receptors releases Cl from inside of immature neurons causing depolarization. The reverse happens when $GABA_A$ receptors are activated in adult neurons. It is shown that the GABA switch is delayed by blockade of $GABA_A$ receptors and accelerated by an increase in activation (Ganguly *et al.*, 2001). However, other mechanisms may also regulate this switch, as it may occur in absence of $GABA_A$ receptor activation (Ludwig *et al.*, 2003).

Contrasting effects of GABA on cell proliferation are reported depending upon the type of precursors and the animal studied. GABA inhibits cell cycle progression of precursors in organotypic striatal slices (Nguyen *et al.*, 2003). It increases proliferation of ventricular zone cells, but decreases proliferation of cells in the sub ventricular zone (Haydar *et al.*, 2000). It decreases DNA synthesis in acute slices (LoTurco *et al.*, 1995), but enhances proliferation of cerebellar granule cell precursors (Fiszman *et al.*, 1999). These differences in the outcomes of GABA effects on cell proliferation may be due to additional factors that work in conjunction with GABA and appear in different cell types at specific times of development. Moreover, substitute mechanisms for the GABA mediated proliferation of neuronal precursors may exist as no obvious changes in the brain structure are noticed following GABA deficiency.

In the developing brain, the postmitotic neurons migrate from their site of origin to the final destination, where they make synaptic connection. Neurotransmitters and an N-type calcium channel regulate migration of immature neurons (Komuro and Rakic, 1998). GABA acting on different GABA receptors acts as motility promoting, accelerating, or stop signal (Lujan *et al.*, 2005). In cultured rat brain slices, $GABA_B$ and $GABA_C$–like receptor activation stimulates migration of neurons from the intermediate and ventricular zones respectively, whereas activation of $GABA_A$ receptors arrests migration of neurons as they approach their target destination (Behar *et al.*, 1996, 1998, 2000, 2001). The role of GABA in the tangential migration of neurons is evident by the fact that a blockade of the $GABA_B$ receptors leads to the accumulation of tangentially migrating interneurons in the proliferating zone in organotypic cultures from embryos (Lopez-Bendito *et al.*, 2003). It is also evident that GABA provides boundary information for the migratory neurons in the ventromedial nucleus of the hypothalamus in mice embryos in a $GABA_A$ receptor-mediated mechanism (Dellovade *et al.*, 2001), and activation of $GABA_A$ receptors by GABA inhibits neuronal migration in the anterior sub ventricular zone and rostral migratory stream of juvenile and adult mice (Bolteus and Bordey, 2004). $GABA_A$ receptor agonist muscimol also inhibits migration of luteinizing hormone-releasing hormone neurons in the embryonic olfactory explants (Fueshko *et al.*, 1998).

GABA also modulates neuronal arbour elaboration and differentiation. Activation of the $GABA_A$ receptor promotes neurite outgrowth and maturation of GABAergic interneurons (Barbin *et al.*, 1993; Marty *et al.*, 1996), and $GABA_A$ receptor antagonists reduce the dendritic outgrowth of cultured rat hippocampal neurons (Barbin *et al.*, 1993). Blockage of Ca^{2+}/calmodulin kinase II (CaMKII) or mitogen-activated protein kinase in cerebellar granule cells reduces the differentiating effects of GABA, suggesting that this process requires a Ca^{2+} influx and an activation of Ca^{2+} dependent kinases (Borodinsky *et al.*, 2003; Maric *et al.*, 2001).

C. GABA, GABA Receptors, and Neurodevelopmental Disorders

Almost all GABA in the brain is synthesized by the decarboxylation of glutamate by two forms of glutamic acid decarboxylase (GAD): GAD_{65} and GAD_{67}. These isoforms are the product of two genes (Erlander *et al.*, 1991). Knockout mouse with deleted GAD_{65} and GAD_{67} have only 0.02% of normal GABA levels in the brain. Double mutants do not survive because of cleft palate (Ji *et al.*, 1999). Histological and immunological studies with fetal and newborn mice show no discernible changes in the brain structures, including in cortical lamination, suggesting that the role of GABA in the early brain development is substituted by other molecules such as taurine acting on a glycine receptor/Cl^- channel (Flint *et al.*, 1998) and glutamate acting at N-methyl-d-aspartate (NMDA) receptors (Behar *et al.*, 1999). Ultra structural studies of the brains of mutant mice and global gene expression analysis may elucidate changes that may occur due to loss of GABA during development. Mice deficient in GAD_{65} mRNA and protein demonstrate conditional fear behavior, spontaneous seizure, lowered threshold for seizure inducing drugs, and increased anxiety-like behavior (Asada *et al.*, 1996; Stork *et al.*, 2000, 2003). GAD_{67} null mouse with only 7% GABA in the brain mRNA develops cleft palate and dies the day of birth with no discernible defects in the brain structures.

In adults, altered GABA levels are reported in several neurological disorders. The GABA level is significantly reduced in the cerebrospinal fluid of patients with Huntington's disease and Alzheimer's disease (Enna *et al.*, 1977). In patients with stiff-person syndrome, prominent and significant decrease in the GABA level is observed in the sensorimotor cortex with a smaller decrease in the posterior occipital cortex (Levy *et al.*, 2005; Wong *et al.*, 2004). GABA concentrations are also lower in the cerebrospinal fluid and blood plasma in unipolar depressed patients (Brambilla *et al.*, 2003; Sanacora *et al.*, 2004). Proton magnetic resonance spectroscopy shows decreased GABA concentrations in the occipital cortex of depressed patients. In contrast, higher levels of GABA are reported in the blood and urine of autistic children (Cohen, 2001; Dhossche *et al.*, 2002). Since GABA

receptors are also expressed in the peripheral tissues (Erdo and Wolff, 1990) many of the behavioral problems of excess GABA may derive indirectly from the abnormalities that may occur in tissues other than CNS. Relevant to this, the self-stimulatory behaviors in autistic children are assigned to higher GABA levels in peripheral tissues (Cohen, 2001).

Altered GABAergic transmission in adults results in anxiety disorder, schizophrenia, and premenstrual dysphoric disorder (Wong *et al.*, 2003). Gene manipulation of different subunits (α_1, α_2, α_5, α_6, β_2, β_3, γ_{2S}, γ_{2L}, and δ) of the $GABA_A$ receptor indicates that basal behaviors of surviving animals in most mutants do not change significantly. This could be because other subunits may compensate for the loss, as manipulation of specific subunit gene expression is associated with altered expression of other subunits (Nusser *et al.*, 1999; O'Meara *et al.*, 2004; Ramadan *et al.*, 2003). Nevertheless, when challenged by drugs or alcohol, many of the mutants behave differently than the control animals, suggesting that compensatory mechanisms work but the threshold level for tolerance in response to stress is reduced in the mutants (Boehm *et al.*, 2004).

Mice deficient in the α_1 subunit of $GABA_A$ receptors display handling induced tremor (Kralic *et al.*, 2005) and α_2 subunit knock-out mice display a lower basal level of locomotion (Boehm *et al.*, 2004). Studies with α_5 knock-out and knock-in mice suggest that α_5-receptor subunits mediate hippocampal dependent learning processes (Collinson *et al.*, 2002; Crestani *et al.*, 2002). Deletion of β_2 subunit results in higher locomotor activity with no signs of motor dysfunction (Sur *et al.*, 2001), whereas β_3 subunit knock-out mice display a wide range of abnormal behaviors, including hyperactivity, poor motor coordination, learning and memory problems, hypersensitivity to sensory stimuli consistent with Angelman syndrome, and a subtype of autism (Buxbaum *et al.*, 2002; DeLorey *et al.*, 1998; Nurmi *et al.*, 2001). Studies with γ_2 heterozygous mice implicate that a γ_2 dysfunction predisposes animals to anxiety (Crestani *et al.*, 1999) which remain undisturbed in δ subunit knock-out mice (Mihalek *et al.*, 1999).

II. Alcohol and Neurodevelopmental Disorders: Role of GABA and GABA Receptors

Neurodevelopmental disorders are the outcomes of suboptimal neurotransmission resulting from anomalies in the CNS development. This may occur due to abnormal proliferation, differentiation, migration, and apoptosis of various precursors that form the heterogeneous tissues of the brain. Functional changes in the brain are derived from altered membrane fluidity and permeability, functions of enzymes, receptors, and signaling molecules, and changes in gene expression patterns in the developing structures. Alcohol influences this entire repertoire in the developing brain, and therefore is a potential neurobehavioral teratogen.

Rodent models are extensively used to understand the mechanisms of prenatal alcohol induced behavioral problems, and a large number of studies support involvements of GABA and the GABA receptor system in the process. Prenatal alcohol exposure during pregnancy and lactation increases GABA levels in the frontal cortex, olfactory bulbs, anterior colliculus, and amygdala in the rat offspring (Ledig *et al.*, 1988). Fetal rats exposed to alcohol during gestation days 1 to 20 also have higher GABA levels in the brain (Maier *et al.*, 1996). Therefore, many of the toxic effects of prenatal alcohol on the developing brains may derive from changes in the extracellular GABA levels and the paracrine actions it mediates through GABA receptors.

Alcohol, by its ability to alter membrane fluidity (Arienti *et al.*, 1993, 1994; Gutierrez-Ruiz *et al.*, 1995), may affect subunit association and stability of various receptors and ion channels (Lovinger *et al.*, 1989; Messing *et al.*, 1986). In neocortical slices from chick, mice, and rat brains and in rat hippocampal CA1 neurons, the $GABA_A$ receptor mediated membrane current is potentiated by alcohol (Reynolds *et al.*, 1992; Weiner *et al.*, 1994).

Gestational alcohol exposure may disturb GABA receptor function by changing the optimum number and affinity of receptors during development. Extensive studies have been done to examine this possibility. Two doses of alcohol on gestation day 8 alter spatial learning of offspring and increase low affinity GABA receptor numbers in the brain (Minetti *et al.*, 1996). Chronic alcohol exposure during gestation increases $GABA_A$-benzodiazepine receptor number and pharmacology in adult guinea pig cerebral cortex (Bailey *et al.*, 1999). Proportions of GABAergic neurons are altered in layers II/III somatosensory cortex of guinea pigs following gestational exposure to alcohol (Bailey *et al.*, 2004). Chronic exposure to prenatal alcohol increases $GABA_A$ receptor $\beta_{2/3}$-subunit protein expression, reduces the growth of the hippocampus, and causes behavioral dysfunction in young adult offspring of guinea pigs that include changes in spontaneous locomotor activity, cognitive deficits, and impaired spatial learning (Iqbal *et al.*, 2004). Another study also reported that the chronic gestational exposure to alcohol in guinea pigs decreases α_1 and $\beta_{2/3}$-subunit protein expression in the cerebral cortex of adult offspring in association with the increase in locomotor activity (Bailey *et al.*, 2001). The cerebral cortical weight is also reduced.

In adult rats, chronic alcohol exposure decreases binding of benzodiazepine with the $GABA_A$ receptor assemblies derived from α_2, α_3, and γ_3 in the cerebral cortex (Mehta and Ticku, 2005). This alcohol sensitivity of α_2, α_3, and γ_2 subunit assemblies in the brain is a consistent observation (Mhatre and Ticku, 1992; Mhatre *et al.*, 1993; Montpied *et al.*, 1991), and it is suggested that this could be due to changes in the trafficking of the subunits to the cell surface as observed for the α_1 subunit in the cerebral cortex following alcohol exposure (Kumar *et al.*, 2003). All these studies strongly indicate that long-term behavioral effects of gestational alcohol may be due to changes in subunit composition, number,

and distribution of GABA receptors in different parts of the brain. There is evidence that the effects of chronic prenatal alcohol on the functions of $GABA_A$ may be different in different brain regions, which would contribute to the complex pattern of cognitive and behavioral dysfunction (Allan *et al.*, 1998).

Exposure to alcohol may change post-translational modification of the $GABA_A$ receptor, affecting allosteric coupling between binding sites and changing receptor function. Intracellular domains of many $GABA_A$ receptors subunits contain a number of consensus sites for serine/threonine and tyrosine protein kinases. Protein kinase A, protein kinase C, tyrosine kinases, cyclic guanosine monophosphate-dependent protein kinase, and calcium/calmodulin dependent protein kinase II modulate $GABA_A$ receptor function by phosphorylating different serine, threonine, and tyrosine residues differently in different subunits of $GABA_A$ receptors (Kumar *et al.*, 2004; Leidenheimer *et al.*, 1991; Stelzer, 1992). For instance, phosphorylation of both serine at the 408 and 409 positions of the β_3 subunit enhances receptor function, whereas phosphorylation of serine at the 408 position reduces channel functions (Moss *et al.*, 1992). Phosphorylation events in association with the differences in subunit composition of receptors may also change receptor function. Phosphorylation of tyrosine residues at the 365 and 367 positions of γ_2 subunit by tyrosine kinase Src with coexpression of α_1 and β_2 causes enhancement of the $GABA_A$ receptor function (Moss *et al.*, 1995).

Several effects of alcohol on the body are due to post translation modification of proteins, such as protein phosphorylation (Mahadev and Vemuri, 1999; Mahadev *et al.*, 2001). Chronic alcohol administration in adult rats alters association of PKCγ with the α_1-subunit containing $GABA_A$ receptors in the cerebral cortical membranes (Kumar *et al.*, 2004). Alcohol-induced changes in the phosphorylation of GABA receptor subunit/s in the embryonic brain therefore may change the receptor functions and the developmental events regulated by these receptors.

During the transition of the excitatory function of $GABA_A$ receptors in the developing brain into a predominantly inhibitory function in the mature brain various subunits undergo functional rearrangements to modify the responsiveness of receptors to GABA (Fritschy *et al.*, 1994; Laurie *et al.*, 1992). This process is believed to be regulated by the receptor activity itself because the receptor maturation is vulnerable to disruption by GABA-mimetic agents and antagonists that disturb $GABA_A$ receptor composition, sensitivity, and function (Belhage *et al.*, 1990; Bitran *et al.*, 1991; Elster *et al.*, 1995). Alcohol shares anxiolytic, sedative-hypnotic, and anticonvulsant properties with benzodiazepines and barbiturates (Reynolds *et al.*, 1992; Weiner *et al.*, 1994), and therefore exposure to alcohol during the GABAergic shift may disturb the normal pattern of subunit repertoire and assembly, causing permanent changes in receptor function. This may cause long-lasting behavioral changes.

Synapse formation may be affected by change in the positioning of GABAergic neurons that may result from disruption of neuronal migration induced by gestation alcohol. It is extensively documented that prenatal alcohol alters the migration of neuronal population in rodents (Hirai *et al.*, 1999; Siegenthaler and Miller, 2004). Anatomical studies of human brains exposed to alcohol during gestation also show ectopic neurons (Clarren *et al.*, 1978). Now, there is evidence that prenatal alcohol reduces GABAergic interneurons in layers II/III of the somatosensory cortex of the adult guinea pig (Bailey *et al.*, 2004), and studies with postmortem human brains provide evidence that defects in GABAergic neurotransmission resulting from disturbances in the neuronal migration and defective lamination may cause schizophrenia and bipolar disorders (Benes and Berretta, 2001).

Alcohol during synaptogenesis and a brain growth spurt, a period equivalent to the third trimester in humans, causes massive cell death in rodent models (Olney *et al.*, 2002a,b). Similar incidence occurs when pups are exposed to NMDA antagonists and GABA receptor agonists (Olney *et al.*, 2002c). Based on these observations, a dual mechanism involving blockade of NMDA glutamate receptor and hyperactivation of $GABA_A$ receptors is proposed for apoptotic death of cells. Potentiation of $GABA_A$ receptor activity in hippocampal cells from postnatal days 1–3 rats with neurosteroid (3α, 5α)-3-hydroxypegnan-20-one or with benzodiazepines mimics previously mentioned phenomenon, suggesting that alcohol-induced death of brain cells in third trimester is mediated by the activation of $GABA_A$ receptors (Xu *et al.*, 2000). Deletion of large numbers of cells at this time of development in the brain by alcohol may diminish brain size and cause behavioral problems (Farber and Olney, 2003).

Concordance amongst GABA receptor β_3 subunit mutation, cleft palate, autism, and FAS (Buxbaum *et al.*, 2002; Culiat *et al.*, 1993; Gordon, 1993; McCauley *et al.*, 2004; Miles *et al.*, 2003; Nanson, 1992) strongly implies that the $GABA_A$ receptor β_3 subunits may be a common mediator for these disorders.

Several effects of alcohol are mediated by $GABA_B$ receptors in adult rodent brains. The alcohol-induced locomotor stimulation, hyperexcitability, and inhibition of specific cerebral Purkinje neurons are modulated by $GABA_B$ receptors (Humeniuk *et al.*, 1993; Mead and Little, 1995; Yang *et al.*, 2000). Moreover, $GABA_B$ receptors modulate alcohol sensitivity of $GABA_A$ receptor mediated inhibitory postsynaptic currents (Wu *et al.*, 2005). These data hint that neurodevelopmental problems caused by alcohol may also be mediated by $GABA_B$ receptors, but experimental evidence on this possibility is currently missing. Data describing involvement of $GABA_C$ receptors in alcohol effects on the brain is also scarce. Studies examining possible involvement of $GABA_B$ and $GABA_C$ receptors in the alcohol-induced neurodevelopmental disorders are sought to elucidate further the role of GABA and the GABA-receptor system in the process.

III. Conclusions

It is evident that GABA and GABA receptors play a significant role in the development of the brain and disturbance in this system during this time of life may result in long-lasting behavior problems. Suboptimal synapse formation resulting from inadequate number and positioning of cells with changed receptor function in alcohol exposed brains is the primary reason for the neurodevelopmental disorders. So far, most studies are done to examine the involvement of GABA systems in this process using a chronic alcohol exposure paradigm. Unfortunately, this approach does not elucidate the mechanisms of alcohol effect on GABA and the GABA-receptor system because development of the brain is a changing process and alcohol may affect this process differently depending upon the time and dosage of exposure. Examining effects of alcohol at specific times of development will facilitate understanding the progression of pathology better.

Abstaining from alcohol is the best way to avoid the evils of alcohol on the fetus but habits of alcohol drinking have not improved since the outcomes became known. Because alcohol's behavioral effects are derived significantly from altered GABA and GABA receptor function, it is imperative that this information be used to develop drugs against alcohol's effects on the developing brain. It is encouraging to know that $GABA_B$ receptor agonists are now used against spasticity, gastro-oesophageal reflux disease, and addiction (Couve *et al.*, 2000; Vacher and Bettler, 2003) and currently, $GABA_B$ antagonists are on trial to improve cognitive improvements of Alzheimer's disease (Couve *et al.*, 2004). These studies assure feasibility of targeting specific molecule of GABA system in the body. Thus, GS39783 that exerts anxiolytic effects by positive modulation of $GABA_B$ receptors in animal models (Couve *et al.*, 2004) may also be tried to prevent behavior problems in subjects exposed to alcohol during development. Similar studies are required with the $GABA_A$ receptor system because a majority of studies show involvements of $GABA_A$ receptors in the alcohol-induced neurodevelopmental disorders. It is promising that compound RY064, which has high a affinity for $GABA_A$ receptor containing α_5 subunit, is being used to antagonize alcohol's neurobehavioral effects in animal models (McKay *et al.*, 2004). This study is yet to be extended in the developmental context.

Vasoactive neuroprotective peptides inhibit prenatal alcohol induced brain malformation in rodent models (Poggi *et al.*, 2003; Spong *et al.*, 2001). Follow up studies examining the effects of these peptides on the GABA system and the behavioral outcomes in offspring are warranted to understand the mechanism better and develop drugs to prevent alcohol's toxic effects on the developing brain. Studies on the effects of other neuroprotective compounds such as RU486, Go6976, secretin, and corticotrophin releasing factor (Ghoumari *et al.*, 2003; Koves *et al.*, 2004; Madtes *et al.*, 2004) on GABA system and behavioral outcomes

of gestational alcohol may aid in finding additional ways to reduce occurrence of neurodevelopmental disorders.

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EFFECTS OF SECRETIN ON EXTRACELLULAR GABA AND OTHER AMINO ACID CONCENTRATIONS IN THE RAT HIPPOCAMPUS

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In 1998, Horvath *et al.* (1998) observed a marked improvement in speech, eye contact, and attention in autistic children 5 weeks after treatment with secretin, during an endoscopic investigation. We investigated the *in vivo* effects of secretin on extracellular amino acids in the rat brain. Studies were carried out on freely moving rats with microdialysis probes in the hippocampus. Amino acids were examined using tandem mass spectroscopy and HPLC/fluorometric detection. Following secretin injection intraperitoneally (8.7 μg/kg), as well as intracerebroventricularly (0.015 μg/0.5 μg/5 μg), considerable increases in microdialysate gamma-aminobutyric acid (GABA) and glutamate levels, as well as a slight increase in microdialysate aspartate levels were observed; other amino acids were not affected. The observed increased microdialysate concentrations of GABA and glutamate following secretin application may contribute to observed effects of secretin in autistic patients.

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I. Introduction

A. The Reawakened Interest in an Old Familiar Hormone

The story that lead to reawakened scientific interest in secretin 90 years after its discovery (the first substance in which the principle of hormonal regulation was identified) began in 1996 when Parker Beck, a three-and-a-half-year-old boy from New Hampshire (USA), was given an injection with secretin to widen the pancreatic duct in line with an endoscopic examination of the pancreas. He had been suffering from autistic symptoms and chronic strong diarrhea since he was 15 months old. The parents of the child witnessed considerable differences in Parker's receptiveness in the days following the injection with secretin and concluded that it must be related to the injection, and thereby discovered the first indications of a possible efficacy of pharmacotherapy on autistic symptoms with secretin. This was, of course, a small glimmer of hope for several families with autistic children; news of which quickly spread through the Internet.

According to the parents, Parker's chronic diarrhea and sleep disturbances became less directly after the aforementioned examination; the young boy, who had been mute since his 15th month, once again began to take up eye contact and developed speech capacities. These developments continued for a period of 3 months, then came to a close, but did not however develop into any kind of deterioration. In total Parker received three infusions with secretin over the period of a year. In 1997 his parents started to treat their son daily with secretin, which they applied transdermally with the help of dimethyl sulfoxide.

Although the described dramatic effect until now has not been able to be secured and proven in controlled clinical studies and therefore remains a topic of considerable debate, nevertheless scientific interest in the neuromodulatory roll of secretin in the CNS has been reawakened.

B. Secretin

The German psychologist Carl F. W. Ludwig (1816–1895) proved in 1851 that secretion from salivary glands is regulated through nerve impulses; around the beginning of the twentieth century, the famous physiologist, Ivan Petrovich Pavlov (1849–1936) investigated the reflex mechanisms that regulate the secretion produced by stomach acids following ingestion. Pavlov developed the well-known theory of the nervous regulation of the salivary and gastrointestinal glands (Henriksen and de Muckadell, 2000).

Taking these studies into account the English doctor and physiologist Ernest Henry Starling (1866–1927) in collaboration with physiologist William Maddoc

Bayliss (1860–1924) undertook an examination into the mechanisms of pancreatic secretion in dogs. In a row of experiments from 1902 to 1904 they showed that the intravenous injection of an extract of duodenal mucosa causes a strong release of bicarbonate and water from the pancreas (Bayliss and Starling, 1902). They postulated that the stimulatory effect of acids in the small intestine on the pancreas secretion is due to the release of a messenger in the upper intestinal mucosa that runs through the circulation system and causes the stimulatory effect on the pancreas. They called this substance "secretin."

In 1905, Bayliss and Starling named this new regulatory principle, where blood is used as the path for transferring information, "hormone." The Greek word "hormao" names a substance that decomposes, stimulates, excites (Henriksen and de Muckadell, 2000).

1. *Isolation and Structure of Secretin*

Secretin was isolated and purified from the porcine intestine for the first time in 1961 by Jorpes and Mutt (1961). They analyzed the sequence of amino acid until 1970 and discovered that secretin is a peptide consisting of 27 amino acids with a molecular weight of around 3000 Daltons (Mutt *et al.*, 1970).

The following chronological order depicts the isolation and sequencing of secretin in pigs (Mutt *et al.*, 1970), cows (Carlquist *et al.*, 1981), humans (Carlquist *et al.*, 1985), in dogs (Shinomura *et al.*, 1987), and the rat (Gossen *et al.*, 1989). Secretin proved to be very much a conservative peptide over the period of the evolution of mammals, where amino acid substitutions only are found in positions 14, 15, and 16.

As claimed by Starling and Bayliss, a precursor-protein with a higher molecular weight was found and identified (Gafvelin *et al.*, 1990).

The relationship of amino acid sequences of secretin to other peptides of the CNS and digestive system showed clearly that it belongs to a family of so-called "brain-gut peptides," to which the following also belong: VIP (vasoactive intestinal peptide), PACAP (pituitary adenylate cyclase activating peptide), GHRH (growth hormone-releasing peptide), PHI (peptide histidine isoleucine) or PHM (peptide histidine methionine), glucagon, GLP-1 (glucagon-like-peptide 1), GLP-2, and GIP (gastric inhibitory peptide) (Ng *et al.*, 2002). These substances, which can be summarized under the formula Sec/Gluc/VIP-super family, all show a structural similarity at the N-terminal end.

The secretin gene of the human was located in the chromosome 11 p 15.5 (Whitmore *et al.*, 2000). Secretin has 25%, or in another context 39%, structural similarity with two other neuropeptides, the hypocretins. These two peptides, that were only found in the dorsal and lateral hypothalamus, are probably not members of the PACAP/Glucagon-superfamily since its identity is found in middle and C-terminal regions, however, not in the N-terminus (de Lecea *et al.*, 1998).

C. The Secretin Receptor

The cloning of a high-affinity secretin receptor was reported first of all by Ishihara *et al.* (1991) who were able to isolate the specific complementary DNA of secretin receptors in the rat out of a cDNA-library of hybrid neuroglial cell line NG 108–15. The nucleotide-sequence analyses showed that it concerned a protein with 449 amino acids and a calculated molecular weight of 49 kDa. Based on these results Ulrich *et al.* were able to isolate exactly the same receptor from a cDNA-library from pancreas cells of the rat (Ulrich *et al.*, 1993).

The secretin receptor contains an N-terminal signal peptide sequence, five possible positions for N-glycosylation, ten cysteine residues that probably lie within the extracellular domain, seven membrane-spanning helices, and three intracellular positions for phosphorylation through PKC; though it belongs to a family of G-protein coupled receptors, interestingly however it shows no significant similarity with other typical receptors of this group, since it discloses the highly conserved sequences of amino acids of the ß-adrenergic family of G-protein coupled receptors, and as a very specific particularization shows a relatively large extracellular domain, which precedes the first transmembrane domain.

With the cloning of the receptors for calcitonin and parathormone and the obvious similarity with the sequence of the secretin receptor it became clear that these receptors belonged to a new branch of the phylogenetic development of the superfamily of G-protein coupled receptors (Ulrich *et al.*, 1998).

Taken together, the receptors of the Sec/Gluc/VIP-family show less than 12% homology with the family of rhodopsin- and ß-adrenoreceptors. The only essential and uniform motifs between the two families are the seven transmembrane helices and the two cysteines that form a disulfide bond between the first and second extracellular domain (Ulrich *et al.*, 1998). The cloning of the human pancreatic secretin receptor showed that it has a 81% uniformity with the pendant of the rat (Jiang , 1995).

The most important functional particularity of the members of the Sec/Gluc/VIP-superfamily lies obviously in their capacity to use two important intracellular signal transduction paths, namely via cAMP as well as through IP_3 (Patel *et al.*, 1995; Sreedharan *et al.*, 1994; Trimble *et al.*, 1987). Secretin creates the synthesis of cAMP by coupling to a G-protein (Fremeau *et al.*, 1986); in addition high concentrations of secretin also stimulate the production of IP_3 with a following intracellular release of Ca^{2+} and the activation of PKC (Trimble *et al.*, 1987).

The strength of specific binding affinity of different ligands on the secretin receptor seems to vary slightly depending on the type of mammal, origin of the organ, and the cell type used. However, radioactive labelled secretin is usually displaced competitively from the receptor in decreasing strength by secretin >

helodermin > PHI (peptide histidine isoleucine) = VIP (Gossen *et al.*, 1990). An opposite succession of the binding affinity was apparent for the VIP-receptor with a decreasing potency in the order VIP > helodermin > GRF > PHI > secretin (Voisin *et al.*, 1991).

Secretin receptors have a relatively high binding affinity for radioactive labelled secretin, where around 50% of the bound 125I-secretin was competitively displaced by 1 nmol/L secretin or by 1 μmol/L VIP (Ulrich *et al.*, 1998). Despite this, the binding behavior shows a certain cross-reactivity between VIP and secretin, which explains the occurrence of high- as well as low-affinity binding sites of both ligands in different organ tissues and the difficulty it caused at the beginning to interpret binding data (Robberecht *et al.*, 1976).

Desensibilization is a ubiquitous phenomenon of receptors of the cell surface and can be achieved through different mechanisms such as decoupling of the receptor from its G-protein, sequestration, and internalization of the receptor or transcriptional down-regulation.

The known mechanism of decoupling of the receptor from its G-protein via phosphorylation of a C-terminal intra cellular domain as a reaction to agonistic stimulation was confirmed for the secretin receptor (Ozcelebi *et al.*, 1995). In addition to this a second, totally independent mechanism of receptor internalization as a reaction to secretin stimulation was reported (Holtmann *et al.*, 1996). VIP-binding to the secretin receptor leads to no evidential desensibilization (Bawab *et al.*, 1991).

D. Effects of Secretin Outside the CNS

Secretin is mainly produced from endocrine cells of the proximal small intestine, the so-called S-cells (Polak *et al.*, 1971). The S-cells belong to the APUD (amine precursor uptake and decarboxylation)-system of the digestion tract and are therefore of neuroectodermal origin. The APUD-system is a peripheral endocrine cell system, whose similarity lies in the production of functional molecules from amine precursors and storage in specific granula. Besides the gastrointestinal tract secretin is expressed in the heart, lungs, kidney, and testicles (Ohta *et al.*, 1992) and in the B-cells of the developing pancreas (Wheeler *et al.*, 1992).

A fundamental function of secretin in the gastrointestinal tract is to stimulate the secretion of water, bicarbonate, and other electrolytes from the epithelium of the ductus pancreaticus as a reaction to the presence of stomach acids and fatty acids in the duodenum (Meyer *et al.*, 1970; Watanabe *et al.*, 1986).

The secretion of bicarbonate is important in the neutralization of the acidic content from the stomach in order to achieve an optimal pH-value for the function of the digestive enzymes of the small intestine. In addition to this the

pancreas secretion in combination with the secretion of the duodenal mucosa creates a protective alkaline layer that protects these from ulcerations (Allen *et al.*, 1986). Secretin also increases the stimulatory effect of cholecystokinine on the acinus cells of the pancreas in the production of an enzyme potent secretion (Rausch *et al.*, 1985) and stimulates pancreas growth (Dembinski and Johnson, 1980).

The effect of secretin in the gastrointestinal tract is not limited to the pancreas. It restrains deflation of the stomach (Jin *et al.*, 1994) and the production of stomach acids (You and Chey, 1987). In the gall bladder it promotes the flow of gall by the opening of Cl^- channels (McGill *et al.*, 1994) and by promoting osmotic water transport in the cholangiocytes through increase of aquaporines in their plasma membranes (Marinelli *et al.*, 1997). Furthermore secretin increases the activity and the concentration of epidermal growth factor (EGF) in the secretion of Brunner's glands in the duodenum (Olsen *et al.*, 1994).

On the kidney it functions anti-diuretically through the activation of an adenylate cyclase on the thick ascending limb of the loop of Henle (Charlton *et al.*, 1986).

On the heart, secretin functions in a positively ionotropical manner and once more promotes the activity of an adenylate cyclase (Rice *et al.*, 1999).

E. Secretin as a Neuropeptide

A functional meaning of secretin as a neuropeptide was increasingly supported in the last few years by anecdotal reports concerning the treatment success in autistic patients, and therefore has been the object of intense speculation. However, even before there were significant and clear indications that secretin had a neuroactive effect in the central and peripheral nervous system.

1. *Central Nervous Expression*

In order to be effective as a physiological neuropeptide, secretin has to be created in specific regions of the nervous system; the expression of mRNA of the secretin precursor peptide in the brain of rats showed at first contradictory results in the data, which on one hand are a clear expression of its existence (Itoh *et al.*, 1991; Whitmore *et al.*, 2000), and on the other hand of its absence (Kopin *et al.*, 1990). Secretin type immunoreactivity in brain tissue of the rat and the pig was already displayed early on (O'Donohue *et al.*, 1981), but could be due to cross-reactivity with other structurally similar peptides. A few years later Fremeau *et al.* (1983) proved the highly specific binding of radioactively labeled secretin to the plasma membrane of cells of different brain regions of the rat. Specific binding was greatest in the cerebellum, intermediate in the cortex, thalamus, striatum, hippocampus, and hypothalamus, and lowest in the midbrain and the pons

(Fremeau, Jr. *et al.*, 1983). Similarly, at first there was no positive proof for the expression of the well-known secretin receptor in human brain tissue (Chow, 1995) or of the rat (Ishihara *et al.*, 1991). Ng, Young, and Chow were able to prove through Northern Blot analysis the presence of secretin receptor transcripts in eight representative brain regions of the rat, namely in cerebellum, cortex, hippocampus, thalamus, hypothalamus, pituitary, brainstem, and striatum, as well as the presence of secretin transcripts in the brainstem and cerebellum (Ng *et al.*, 2002). They also assumed the expression of secretin beneath detection limits in specific neurons of other brain regions. Rindi for example reports the selective expression of marked secretin in the serotoninergic neurons of the dorsal raphe nuclei in mice (Lossi *et al.*, 2004; Rindi *et al.*, 2001).

Studies in rats gave evidence that secretin and its receptor are regulated during postnatal brain development. Tay *et al.* (2004) observed stronger expression of both in weeks 3 to 5 as compared to week 2. The areas of major expression were the cerebellum, the central amygdala, hippocampus, area postrema, and the nucleus tractus solitarius.

2. *Central Nervous Effects*

The oldest description of central nervous effects stems from Fuxe, who pointed out the increase of dopamine turnover in the eminentia mediana, which coincides with a decrease in the prolactin secretion following intracerebroventricular (i.c.v.) injection of secretin; in connection with the observation of further representatives of this peptide family he suspected a hormonal gastrointestinal-hypothalamic-pituitary loop (Fuxe *et al.*, 1979). 1 μg of i.c.v.-injected secretin lead to the stimulation of the hypothalamic tyrosine hydroxylase and a decrease in plasma-prolactin and LH-levels, 5 μg on the other hand increased the concentration of prolactin, but did not change anything in the other measured parameters (Babu and Vijayan, 1983).

Samson indicated secretin-like immunoreactivity in regional brain extracts of the rat, especially in pituitary, hypothalamus, epiphysis, and the septum; apart from this he describes the increase of serum prolactin levels on a high dose application of secretin i.v. (10 μg), as well as a dose-dependent prolactin stimulation *in vitro* in cells of the anterior hemipituitary through synthetic secretin (0.31 μg/ml). However, i.c.v. injection of secretin (0.1–10 μg) led to a restriction of the plasma prolactin level (Samson *et al.*, 1984).

Charlton *et al.* (1983) noticed under i.c.v. application of secretin (5 μg in 5 μL of solution) an increase in defecation, but decreased novel-object approaches and open-field locomotor activity in rats, as well as a change in the respiration rate of anaesthetized animals. None of these effects were able to be shown after i.p. injected secretin. Babarczy *et al.* (1995) described an influence of 0.01 μg i.c.v.-applied secretin on the pain sensitivity of rats through the development of morphine tolerance on one dosage of morphine, but not in regular doses.

VIP and secretin induced the genetic expression of tyrosine hydroxylase in PC12-cells through independently antagonizable, yet not additively effective mechanisms. They were in both cases proteinkinase-A- and adenylate cyclase dependent mechanisms (Roskoski *et al.*, 1989; Wessels-Reiker *et al.*, 1993). The tyrosine hydroxylase is the velocity-defining enzyme in the synthesis of the catecholamines. A modulation of this enzyme was also seen in the upper ganglion of the rat (Ip *et al.*, 1982).

Van Calker *et al.* (1980) described a strong cAMP-stimulation through secretin in cultivated brain cells of the mouse, which mainly consist of glioblasts. VIP showed, in relation to this parameter, a minimal stimulatory potency. Both effects could be inhibited with somatostatin, the first one only with secretin-(5–27).

Further indications of induced cAMP-accumulation in brain slices of these two substances were found by Fremeau (Fremeau *et al.*, 1986).

Very recent electophysiological examinations by means of patch clamp technique on neurons of the nucleus tractus solitarius of the rat have shown that an application of secretin to this region causes a non-selective increase in conductivity of cations causing depolarization of these neurons; this modulatory effect apeared concentration dependent and was not blockable by tetrodotoxin (Yang *et al.*, 2004a).

3. *Interaction of Secretin and GABA in the Cerebellum*

Yung *et al.* (2001) demonstrated the interaction between GABA and secretin and the role of secretin as a direct neuromodulator in the CNS through electrophysiological measurements. First, this group was able to show the expression of secretin and its receptors in the cerebellum of the rat using Northern Blot analysis. In-situ hybridization showed immunoreactivity of secretin, which was confined to the soma and the dendrites of Purkinje cells of the cerebellar cortex, whereas immunoreactivity of the secretin receptor could be shown in Purkinje cells as well as in smaller cells that were positively identified as basket cells. Electrophysiological examinations using whole cell patch clamp technique on Purkinje cells showed at first no change in the resting potential on secretin (3–300 nM), although receptors had been found. After that, the Purkinje cells were stimulated by activation of afferent fibers or interneurons. Resulting was a longlasting (>30 min) increase of the amplitude and frequency of spontaneous and evoked IPSCs after a brief exposition to secretin (3–5 min with 3–300 nM solution), whereas the measured IPSCs through stimulation of the parallel fibers showed no change. The IPSCs were found to be largely mediated by $GABA_A$-receptors. The observed effect of secretin started with a delay of 1–2 min, but lasted for more then 30 min. The first effect backs the theory that secretin increases the probability of vesicular release from presynaptic terminals of GABAergic basket cells in the cerebellum, whilst the aforementioned consideration of this thesis does not

necessarily contradict it (Yung *et al.*, 2001). Because secretin receptors had been shown to exist on basket cells as well as on Purkinje cells, the existence of a possible postsynaptic modulation mechanism such as the modulation of $GABA_A$-receptor sensitization was examined through measurement of IPSCs, created by a constant, exogenous utilized amount of GABA. In this instance, unexpectedly, a reduction of the response amplitude under the presence of secretin was observed.

In general this functional knowledge, combined with the detection of secretin in the somatodendritic region of the Purkinje cells and the evidence of receptors on the same cells, as well as in GABAergic basket cells, suggests that secretin may act as a retrograde messenger in the cerebellum of the rat. In summary they observed that secretin facilitates evoked, spontaneous, and miniature IPSCs recorded from cerebellar Purkinje cells. They suggested that secretin is released from the somatodendritic region of Purkinje cells and could serve as a retrograde messenger modulating GABAergic afferent activity.

F. Secretin and Autism

In an uncontrolled case study carried out in 1998 Horvath *et al.* (1998) described three patients with autistic symptoms and chronic diarrhea, who not only showed an increased pancreatobilar answer to i.v. applied secretin, but also demonstrated within 5 weeks a marked improvement in their gastrointestinal symptoms, as well as drastic behavioral changes and an increase in their expressive speech capacity. The authors speculated that these clinical observations could be evidence of a connection between gastrointestinal and cerebral function in autistic persons. Horvath noticed that empirical studies were necessary in order to substantiate the connection further and emphasized the importance of placebo control in such studies.

On October 7, 1998, Dateline NBC wrote a report on Parker Beck's dramatic behavior changes, one of the boys described in the Horvath study. The case carried considerable weight for the media, Internet, and amateur press. Word spread immediately of what was in fact the success of an uncontrolled treatment and quickly, worldwide, parents began to demand secretin infusions for their autistic children. It is estimated that thousands of children were given secretin injections in an off-label use following this highly publicized case, which had not been backed by any profound empirical evidence. There were of course those who warned of the dangers and who threatened to obstruct the further therapeutic use of secretin in absence of an appropriation by the FDA (Federal Drug Administration, USA) until studies into the effectiveness of the therapy were carried out.

There was considerable concern about the possibility of a triggering of allergic reactions to multiple injections of porcine secretin, as well as about the danger of an immunization through the application of porcine secretin where an autoimmune activity to the body's endogenous peptide could possibly result (Esch and Carr, 2004). So in the years to follow, numerous randomized double-blind studies attempted to clinically substantiate the treatment effect.

Several studies included persons who had been diagnosed within the spectrum of autistic illnesses ("autistic disorder," "pervasive developmental disorder," or "pervasive developmental disorder not otherwise specified"). In eight of the studies, porcine secretin was used; in two, synthetic human secretin; and in one of the studies biological as well as synthetic porcine secretin was compared to placebo. Ten studies examined a single secretin dose; in one study secretin was injected twice. All studies used different established tests as parameters to determine autistic modes of behavior, including spoken, cognitive, and pro-social capabilities. In addition to the records to be filled in by trained doctors, in some studies the parents' considerations were taken into account using specialized questionnaires. Detailed, study-specific listings of the instruments used, as well as the data relating to the results, have not been included in this work. For a detailed and retrospective overview and discussion of the aforementioned clinical studies we refer to Esch and Carr (2004).

None of the studies were able to reproduce the dramatic treatment success within a larger population under clinically controlled specifications. One critique in reference to some of the studies was that some test persons were not interrupted from other common pharmaco therapies currently in use (Dunn-Geier *et al.*, 2000; Sandler *et al.*, 1999).

A specific effect that is described in several of the studies concerns the fact that within the secretin and the control groups a certain number of children showed improvements in their autistic behavior, which however did not differ by statistical significance. A possible explanation for this phenomenon could possibly be an effect of habituation of the children to the test situation; as well as a strong expectation effect (Coniglio, 2001; Corbett *et al.*, 2001; Dunn-Geier *et al.*, 2000; Sandler *et al.*, 1999; Unis *et al.*, 2002). Lightdale *et al.* (2001) tried to replicate Horvath's results, and treated 20 autistic children with gastrointestinal problems with porcine secretin in an uncontrolled, prospective study. Contrary to the estimations of the doctors in which no significant treatment effect could be recognized, 70% of the parents reported medium to strong changes, and 85% were of the opinion that their child would benefit from a further secretin infusion. In a controlled study that purely had the aim of evaluating the parents' estimation of the behavioral improvements of their autistic children, it became clear that the parents were not able to distinguish between their child having had a secretin or placebo injection (Coplan and Souders, 2003).

Maybe the unusual background of these studies biased parents' assessment. The circumstances under which several of them had wanted to take part in a secretin study are heavily emotionally burdened and laden with expectations of success (Esch and Carr, 2004).

In addition to this there was a further more unusual aspect to be taken into account in these tests, which was that the impulse to begin with these studies was driven strongly by media coverage, and that again and again anecdotal reports were issued, as well as singular case descriptions from clinics about the positive treatment effect of intravenous as well as transdermally applied secretin (Lamson and Plaza, 2001).

Because Horvath had indicated the presence of gastrointestinal co-morbidity in addition to the autistic symptoms in the first description, some of the studies previously described (Kern *et al.*, 2002; Roberts *et al.*, 2001; Unis *et al.*, 2002) included a subgroup analysis of children with gastrointestinal dysfunction. The expectation was to possibly identify a subgroup of secretin responders in what was an extremely heterogenic patient group of autistic persons; yet this did not bring about any significant differences.

Only in Kern's study were there any notable positive effects of a secretin dose in the comparison between autistic children with active chronic diarrhea and those without gastrointestinal problems. The patients with chronic obstipation or chronic diarrhea in a period of remission were not included; the number of those within the group with chronic diarrhea was limited, unfortunately, to only five individuals. These showed, under treatment with secretin, a significant reduction in anxiety, agitation, crying, hyperactivity, non-compliance, lethargy, and social withdrawal, as well as a decrease in stereotypical behavior and a more adequate use of language (Kern *et al.*, 2002).

The question of whether children with autism suffer more regularly from gastrointestinal problems than those of the same age without autism is a highly debated topic. Horvath and Permann described, in a study conducted on twins in 2002, the increased occurrence of gastrointestinal inflammation in autistic persons, fewer digestion enzymes, a decreased sulfation capacity of the liver, and a higher secretory answer to secretin stimulation tests (Horvath and Perman, 2002). D'Eufemia reported a changed intestinal permeability of 9 of 21 (43%) randomly selected autistic patients, yet in none of the 40 control persons (D'Eufemia *et al.*, 1996). Furlano described specific histological changes of the intestinal wall in autistic patients with gastrointestinal symptoms, which coincides with impaired epithelial glycosaminoglycan composition, and which seems to be histologically different to other inflamatory intestinal illnesses (Furlano *et al.*, 2001).

In contradiction to this an analysis of public related data of a research data bank in Great Britain showed the same prevalence of 9% gastrointestinal disorder within the population of autistic as well as non-autistic children (Black *et al.*,

2002). It is therefore clear that as yet there is no true consensus regarding the connection between the development of autistic and gastrointestinal disorders.

Perhaps it would therefore be more promising to undertake a further clinical study on a larger population of autistic children with active chronic diarrhea to identify a secretin sensitive subgroup. The discussion whether secretin leads to peripheral improvements in the gastrointestinal function or whether it acts directly in the CNS would of course continue.

In the absence of a causal medical therapy for autistic disorders there is an extremely wide spectrum of therapeutic attempts by parents who look for help, which is promoted in Internet forums and by organizations involved. The "autism research institute" has been collecting, amongst other things, data on the experiences of parents who have been involved with interventions of all types since 1967. The published chart (http://www.autismwebsite.com/ari/treatment/form34q.htm) enables an overview of the non-standardized assessments of parents. Remarkably, the top positions in regard to the positive influence on the development of the illness are taken up with some diets, vitamins, and enzyme substitution. With regard to the group of drug therapeutics secretin shows a superior treatment profile to almost all other drugs, except Risperdal.

The company Repligen in Needham, Massachusetts, has attained the US-patent right to the treatment of secretin in autistic patients from Victoria Beck and Bernhard Rimland from the Autism Research Institute, USA. The company planned the production and marketing of a synthetic form of human secretin. The FDA (Food and Drug Administration, USA) agreed in advance to quickly give Repligen the right to use secretin in the treatment of children with autism.

In February 2002, five animal tests were conducted into the toxicity of multiple secretin injections (Adis International, 2002).

Phase-II-studies in the USA showed with regard to 3–4-year-old autistic children, yet not in 5–6-year-olds, an improvement in social interaction in accordance with the Autism Diagnostic Observation Schedule.

A later phase-III-study on 132 children between 2 and 5 years old with six injections over a period of 18 months was not able to give evidence of the superiority of an intravenous secretin treatment over one with placebo (www.repligen.com). Details concerning study design as well as the attempt to identify subgroups of secretin responders have yet not been published. Autistic components are also regularly apparent in other psychiatric illnesses, as for example in schizophrenia. A case study by Alamy describes a temporary, substantial improvement in autistoid symptoms of schizophrenic patients to an adjuvant injection of secretin (Alamy *et al.*, 2004). In order to investigate a possible benefit on social behavior in other psychiatric illnesses further clinical studies are being undertaken by Repligen concerning anxiety disorders and negative symptoms in schizophrenia (www.repligen.com).

G. Neuropathologic Findings in Autism

Through post mortem examinations on autistic brains and *in vivo* examinations of autistic people by MRI, evidence of neuroanatomic changes in the following brain regions were found:

- Hippocampus (Acosta, 2003; Aylward *et al.*, 1999; Raymond *et al.*, 1996; Saitoh *et al.*, 2001)
- Amygdala (Aylward *et al.*, 1999)
- Cerebellum (Acosta , 2003; Fatemi *et al.*, 2002; Purcell *et al.*, 2001)
- Corpus callosum (Egaas *et al.*, 1995)
- Regions of the cortex (Casanova *et al.*, 2002)

Casanova *et al.* (2002) detected changes in the mini-columnal structure of the cortex in autistic persons in post mortem examinations of brain tissue from Area 9 of the prefrontal cortex and the Areas 21 + 22 of the temporal lobe of autistic persons.

Cell columns were increased, but were smaller and less compact in their cell structure and had less peripheral neuropil space (Casanova *et al.*, 2002). In this histological compartment fibers of inhibiting interneurons are evident (Casanova *et al.*, 2003).

By using MRI, Courchesne investigated the growth of the brain in autistic children in comparison to healthy children and discovered an early overly large growth followed by a later abnormally slow growth in volume. From the ages of 2–4 there was a hyperplasia of the grey and white substance of the cerebrum and of the white substance of the cerebellum (Courchesne *et al.*, 2001).

Corresponding results were also attained in a study by Aylward (2002). In older autistic persons a diminished size of the cerebellum (Fatemi *et al.*, 2002) and a smaller total number of Purkinje cells were recorded (Acosta, 2003). In connection with this it is also notable that research in the last few years has lead to evidence that the cerebellum is also involved not only in motoric, but also sensory, cognitive, and affective brain functions (Allen and Courchesne, 2003; Ciesielski and Knight, 1994).

Purcell *et al.* (2001) compared the gene expression in the cerebellum of autistic patients with that of healthy individuals. It was discovered that there was a higher expression of a transporter for excitatory effective amino acids (EAAT1) and for an AMPA-sensitive non-NMDA-Glutamate receptor. The density of these receptors has been proved however to be decreased. The examination of post mortem brain tissue of older autistic persons has the disadvantage that it is not possible to recognize which of the apparent changes are of a primary and which are of a secondary nature.

By using MRI-examinations, Saitoh *et al.* (2001) described the presence of significantly decreased cross-section surfaces of the dentate gyrus (comprising the

dentate gyrus and CA4-region) in the hippocampus, however there was no decrease measurable in the totality of the subiculum and the CA1-CA3-regions of autistic persons. Abnormalities of this type are typically to be put down to an increase in the pyramidal cell thickness, reduction in neuron size, and a diminished dendritic tree (Acosta, 2003; Raymond *et al.*, 1996). Using the *in vivo* study it was possible to show that neuropathological changes already were apparent by the time of the clinical manifestation of the symptoms (Saitoh *et al.*, 2001).

Eriksson and colleagues (Eriksson *et al.*, 1998) were able to prove that the neurogenesis of the corn cells in the dentate gyrus is not limited to the pre- and peri-natal life span, but in fact is still evident in adults. The existing neuropathological hippocampal correlates that already prevail whilst the individual is alive and the persistence of autistic symptoms in later life suggest that autism is connected with a non-remitting impairment of hippocampal neurogenesis.

II. Aim

The aim of the current study was to determine the effects of secretin on extracellular amino acid concentrations *in vivo*. To this end, microdialysis was performed in the hippocampus of freely moving rats. Our major interest was in the effects of secretin on glutamate and GABA, given their proposed role in autism (Blatt *et al.*, 2001; IMGSAC, 2001; Muhle *et al.*, 2004b; Purcell *et al.*, 2001). In a row of experiments secretin was applied intraperitoneally; herein it is highly probable that a vagal, as well as a hematogenic transmission into the CNS also took place. Following on from this secretin was injected intracerebroventricularly into different concentrations, in order to compare the found effects with that of a direct neuromodulation.

The hippocampus was chosen as the area for examination, since it is involved in selective attention processes, learning and memory functions, and the creation of motivational states. As a periventricular organ it can be immediately accessed by i.c.v.-applied secretin, and there are indications of the presence of secretin receptors in the hippocampus (Karelson *et al.*, 1995; Ng *et al.*, 2002). Besides this there are signs of a hippocampal dysfunction in autistic patients; neuropathological findings relate, amongst others, to the area dentata (Saitoh *et al.*, 2001).

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III. Materials and Methods

A. Animals and Drug Treatment

In the first series of experiments 30 clinical units of secretin (Secrelux, Goldham) or 8.7 μg secretin pentahydrochloride/kg of body weight were administered i.p.; control animals received an equal volume of saline solution. In the second set of experiments secretin was applied i.c.v. in 5 μL, infused during 2.5 minutes in three doses: 0.015 μg, 0.5 μg, 5.0 μg.

B. Surgery and Microdialysis Procedure

Microdialysis was performed with CMA/12 microdialysis probes (length: 1.0 mm; o.d.: 0.24 mm, Carnegy Medicine, Sweden). The probe was implanted together with a guide cannula under isoflurane (2%) anesthesia into the left hippocampus, as previously described (Clement *et al.*, 1998). This was done according to coordinates given by Paxinos and Watson (Paxinos and Watson, 1982): A:5.2 mm; L:2.0 mm; V:6.5 mm, and fixed with two screws and dental cement. Probes were each used three times. For i.c.v. application, a guide cannula was implanted in the lateral ventricle according to the following coordinates: A:7.2 mm; L: 1.7 mm; V:4.5 mm from the top of the cortex. Positions of the microdialysis-probe and the cannula were controlled histologically.

Microdialysis experiments were carried out in freely moving rats (CMA/120 freely moving system, Carnegy Medicine, Sweden) directly after implantation of the probe. Ringer's solution (NaCl 140 mM; KCl 3.0 mM; $CaCl_2$ 1.2 mM; $MgCl_2$ 1.0 mM) was used for perfusion at a flow rate of 1.25 μL/min (CMA/100, Carnegy Medicine, Sweden), and 25 μL fractions were collected; outlet tubing volume was 3 μL. *In vitro* recoveries at room temperature were found to be about 6% for GABA and 9% for aspartate and glutamate. After six fractions had been collected, secretin or saline were applied i.p. and sampling continued for a further two hours. At the conclusion of the experiment, the rat was euthanized with CO_2. To verify probe placement, rats were decapitated and the brain removed and frozen. Serial coronal sections (thickness: 20 μm) stained with cresyl violet were used for localization according to Paxinos and Watson (Paxinos and Watson, 1982).

C. Assay of Amino Acids

To assess amino acid concentrations (except GABA, glutamate, and aspartate), 200 μL of a solution of deuterated amino acids (internal standards) in methanol was added to 7 μL of dialysate and the mixture evaporated to dryness

at 55 °C under a stream of nitrogen. The residue was heated for 15 min at 65 °C with 120 μL butanolic hydrochloric acid (3 M). After evaporation to dryness, 200 μL acetonitrile/water (1:1 v/v) containing 0.025% formic acid was added and the amino acids estimated as their butylesters by tandem mass spectrometry using a PE Sciex API 365 instrument employing the neutral loss (102 Dalton) mode (Chace *et al.*, 1993). Glutamate, GABA, and aspartate were assayed by HPLC with fluorescence detection (Gerlach *et al.*, 1996), using the precolumn derivatization method with ortho-phthaldialdehyde (OPA) and an automatic system from Kontron Instruments (Neufahrn, Germany) consisting of a 325 pump, a 465 autosampler, a SFM 25 fluorescence detector, and a computing integrator equipped with a 450-MT2 data system. Excitation and emission wavelengths of the fluorescence detector were set at 330 nm and 450 nm, respectively. 5 μL of microdialysate was used for derivatization with 30 μL of OPA reagent (Grom Analytik, Herrenberg, Germany) diluted 10 times with 1M borate buffer (pH 10.7). 20 μL of this reaction mixture was injected directly into the HPLC system. Concentrations were calculated from peak height with the aid of external standards.

D. Statistical Analysis

Concentrations were not corrected for in vitro recovery. The mean concentration of the six samples before drug application was arbitrarily defined as baseline control (= 100%). All values are expressed as percentages of control: S.E.M. Microsoft Excel 8.0 and SPSS were employed for statistical analysis by non-parametric repeated measurement, one-way analysis of variance on ranks and paired Student t-test. The statistical level of significance was set at $*p < 0.05$, $**p < 0.01$. Areas under the curve were also calculated and compared by paired Student t-test.

IV. Results

Systemically administered secretin (8.7 μg/kg i.p.) was followed by an increase in extracellular GABA and glutamate levels, persisting up to the end of recording two hours after application, while the saline-treated group showed a slight decrease over time (see Fig. 1A and B). Other amino acids investigated were not affected by secretin application, with the exception of aspartate, showing a slight increase after secretin application.

The i.c.v. application of secretin led to a dose-dependent increase of GABA (see Fig. 2) and glutamate (see Fig. 3), as compared to saline, with the highest effects, $p < 0.01$, induced by the medium dose of 0.5 μg for both neurotransmitters.

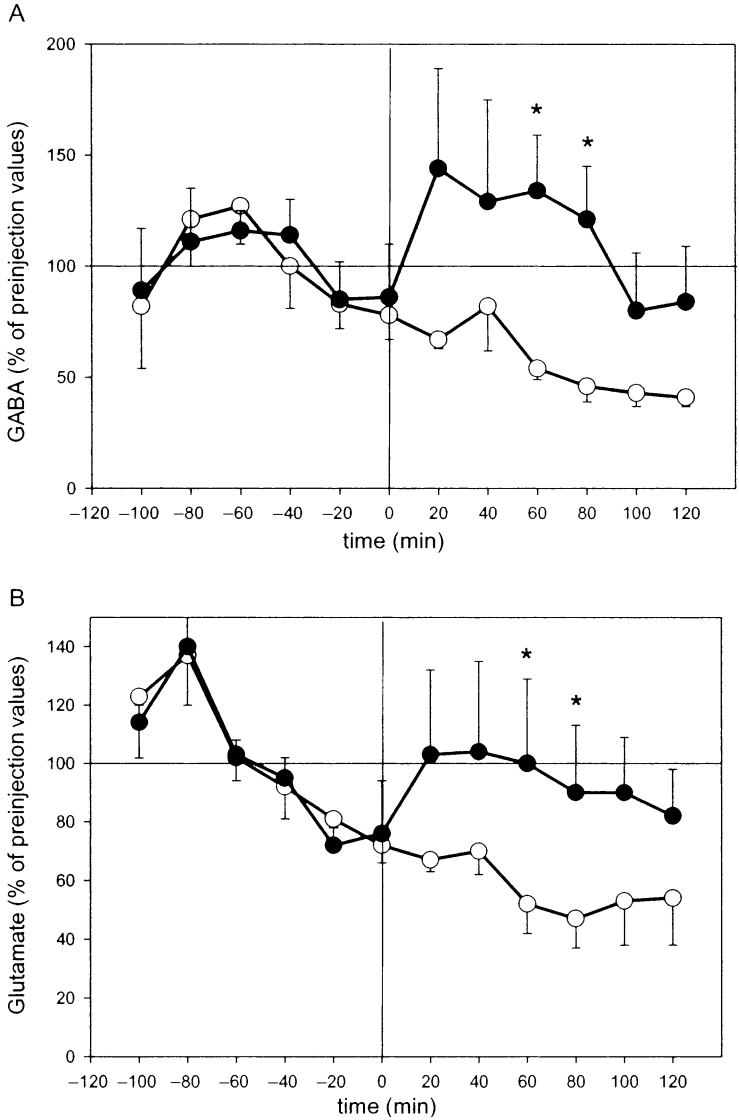

FIG. 1. Time-dependent effect of secretin (8.7 μg/kg i.p., filled circles) and saline (open circles), administered i.p. at 0 min, on extracellular concentration of GABA (A) and glutamate (B) in rat hippocampus. Implantation of microdialysis probes and analysis of the samples by HPLC were performed as detailed in Materials and Methods. Data are presented as mean 1± SEM; n = 5. *$p < 0.05$ student t-test.

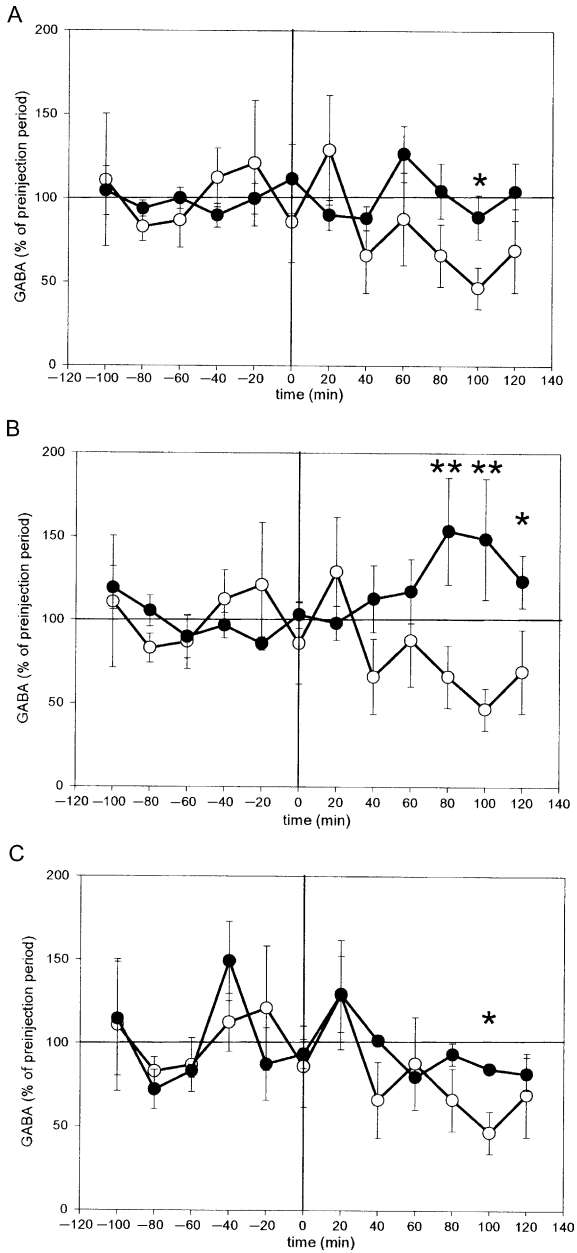

FIG. 2. Time- and dose-dependent effect of secretin (filled circles) applied and saline (open circles), administered i.c.v. in 5 μL at 0 min. A:5.0 μg; B:0.5 μg; C:0.015 μg, on extracellular concentration of GABA in rat hippocampus. Implantation of microdialysis probes and analysis of the samples by HPLC were performed as detailed in Materials and Methods. Data are presented as mean 1 $\pm$ SEM; n = 5. *p $<$ 0.05, **p $<$ 0.01 student t-test.

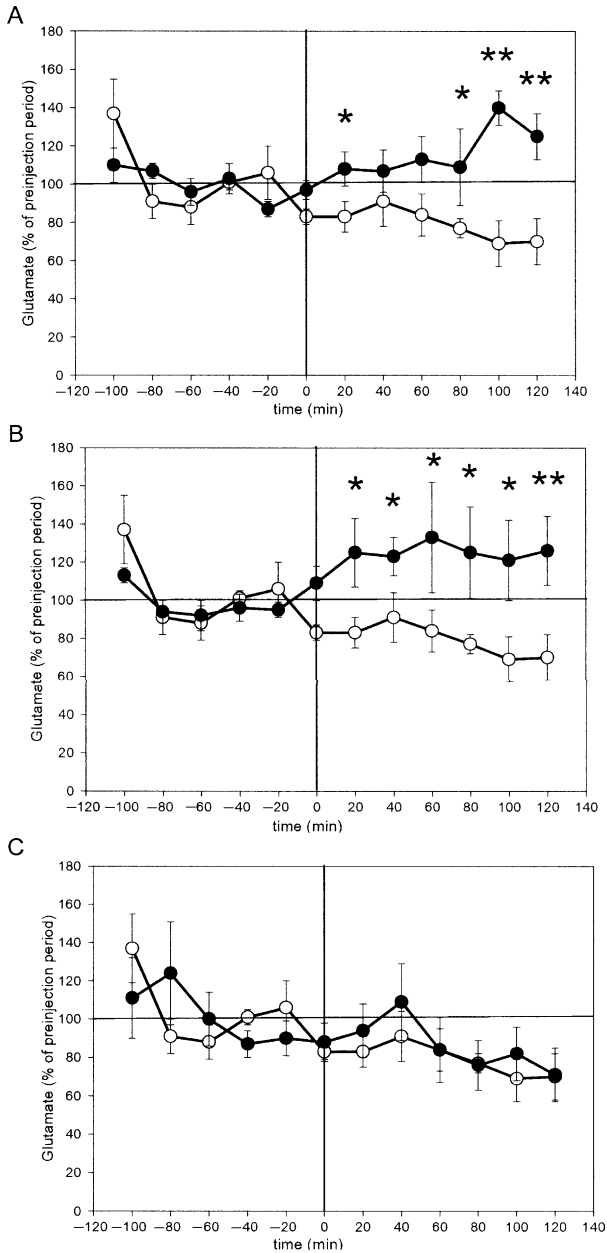

FIG. 3. Time- and dose-dependent effect of secretin (filled circles) applied and saline (open circles), administered i.c.v. in 5 μL at 0 min. A:5.0 μg; B:0.5 μg; C:0.015 μg, on extracellular concentration of glutamate in rat hippocampus. Implantation of microdialysis probes and analysis of the samples by HPLC were performed as detailed in Materials and Methods. Data are presented as mean 1± SEM; n = 5. *p < 0.05, **p < 0.01 student t-test.

V. Discussion

More than 150 years after its discovery as a hormone of the gastrointestinal tract secretin has once again become an object of scientific discussion in its role as a neuropeptide. The interest in research into the neuromodular potency of this peptide hormone was reawakened owing to the astounding report issued on the treatment success of secretin on autistic children (Horvath *et al.*, 1998). First indications of the possible use of secretin were already discovered in the 1970s (Fuxe *et al.*, 1979). The effects of secretin on the hippocampus of the rat in the metabolism of amino acids were examined in the present paper. The main focus was placed on glutamate and GABA, which are the two most important brain transmitters in mammals. Secretin was applied intracerebroventicularly in three different dosages, in order to measure the direct neuromodulatory effects of the peptide. The results show an influence of extracellular concentrations of glutamate, aspartate, and GABA in the hippocampus using secretin; the extracellular concentration processes of other measured amino acids, that is, phenylalanine, alanine, methionine, glycine, ornithine, arginine, tyrosine, valine, leucine/isoleucine, and citrullin were not significantly affected.

The stimulatory effect on the three transmitter substances was strongest in a mean dosage of 0.5 μg secretin i.c.v. Curve progressions of glutamate show a prompt and sustained concentration increase up to the end of the experiment; the process of GABA concentration also shows a late but significant elevation. Moreover a weaker effect on the aspartate concentration was identifiable.

Furthermore the high dosage application of secretin (5 μg) demonstrates the described effect on the glutamate/glutamine and also selectively on the glutamate concentration, whereby a noticeably stronger influence in the later phase of the experiment in contrast to the early phase is especially striking. The effect of the GABA concentration can only be assessed as very weak and the influence of aspartate is no longer traceable.

An unusual aspect of the findings of this chapter is the more or less simultaneous rise of glutamate and GABA, since both of these brain transmitters are primarily associated with antagonistic effects on the CNS. Glutamate is the main transmitter of corn cells of the hippocampus, which create the moss fiber system in their totality and as such give excitory impulses of the glutamatergic pyramidal cells of the cornu ammonis, whilst however they also have collaterals to interneurons. Contrary to this most hippocampal interneurons, particularly the basket cells, are involved in GABAergic inhibition of the pyramidal cells, yet there are also other interneurons involved (Freund and Gulyas, 1997).

There are several different ways in which both these main transmitter systems in the hippocampus can interact with one another and thereby influence one another's release. Whilst GABA via $GABA_A$-receptors (predominantly in

somatodentric regions) and $GABA_B$-receptors (predominantly located presynaptically) can have an inhibitory effect on the release of glutamate, almost every inhibitory interneuron in the hippocampus conversely gets synaptic input from the main cells. This regulation is rather complex, as it exhibits localization and receptor specific differences. Both synaptic NMDA- and non-NMDA receptors of AMPA type as well as metabotrophic receptors of the somatodentric region have a stimulatory effect on the release of GABA in the hippocampus. There are indications of a glutamatergic inhibition of interneurons on presynaptic Kainate receptors and terminal located metabotrophic glutamate receptors (Vizi and Kiss, 1998).

On the level of the reuptake there are also possibilities for an interaction between GABA and glutamate. Since both are essentially taken up into the synaptic endings by an Na^+-dependant, active co-transport, an intracellular strongly rising Na^+-concentration uptake induced by the one molecule can promote the inversion of the carrier-transport of the other (Vizi and Kiss, 1998).

Theoretically, in this work, the measured extracellular increase of one of the measured parameters could be the result of an increase of the other, in contradiction to this however there are several microdialysis studies on the hippocampus of the rat, that only describe an independent stimulation of the transmitter system. For example Giovanni *et al.* (2001) examined the influence of locomotor activity associated with investigatory and habitual behavior to new surroundings on the acetylcholine-, glutamate-, and GABA-concentrations in the hippocampus in rats. The experiments showed a lightly positive correlation between locomotor activity and GABA-concentration, yet no significant influence of the glutamate-concentration. The measured extracellular concentrations in microdialysis experiments of functional amino acids mirror the relationship of transmitter release and reuptake in nerve endings and glial structures (Herrera-Marschitz *et al.*, 1996). In regard to glutamate and GABA there are especially controversial discussions taking place about the real origin of these particular microdialytic transmitters, since they are not, as is classically demanded for proof of the synaptic origin of neurotransmitters, able to be inhibited with certainty by TTX-application and Ca^{2+}-depletion of the tissue (for further discussion see Timmerman and Westerink, 1997). It is therefore unclear whether the measured parameters mirror the real neuronal transmission, an influence, or even an inversion of the neural and/or glial reuptakes, or overall changes in the neurotransmitter metabolism.

There are certainly three conceivable ways in which secretin can influence the CNS: auto, that is, paracrine neuro-neuronal, endocrine by manner of blood paths of peripheral organs to the CNS, and lastly over the nervus vagus. The secretin receptor, as well as secretin itself, is functionalized in the CNS by neurons. The distribution of both molecules does not seem to show disregarded type-specific differences: humans and rats demonstrated an extremely similar

immuno reactivity to secretin in the CNS, that is in the Purkinje cells of the cerebellar cortex, the central cerebellar nuclei, the pyramidal cells of the motor cortex, and in primary sensory neurons; in addition to this there was evidence of presence in the hippocampus and amygdale of the human and neurons of the auditory system of the rat. Secretin was only traceable in the spinal ganglion of the cat (Koves *et al.*, 2004). Ng and colleagues proved, using Northern Blot, the presence of secretin receptor transcripts in the brain of the rat, in the cerebellum, cortex, hippocampus, thalamus, hypothalamus, hypophysis, brain stem, and striatum. However secretin transcripts themselves were only found in the brain stem and cerebellum (Ng *et al.*, 2002). Transgene mice express secretin in the serotoninergic neurons of the dorsal raphe nuclei (Rindi *et al.*, 2001).

Even if the expression of the known secretin receptor in the hippocampus of the rat was described, one must be reminded of the fact that secretin shows a substantial cross-reactivity with other members of its peptide family, and can therefore also bind to other receptors, especially to the VIP-receptor. It is known that VIP can strengthen the GABA-release in the hippocampus through presynaptic receptors (Wang *et al.*, 1997). The VIP receptor 1 (VPAC 1) also reacts, besides VIP, to secretin and PACAP with an increase of cAMP (Vaudry *et al.*, 2000). VPAC 1 and VPAC2-receptors are present in the brain of the rat, among other structures in the hippocampus (Joo *et al.*, 2004). The signal path of the VIP- and PACAP-receptors also works, amongst other means, through an activation of the adenylate cyclase (McCulloch *et al.*, 2002). It is therefore also feasible that secretin was also responsible through the VIP-receptor for the increased release of GABA and/or glutamate observed in this study.

Through the intraventricular application it was confirmed that the effects of secretin took place directly in the CNS. Yung *et al.* (2001) first of all created a hypothetical model based on their electrophysiological measurements on the Purkinje cells in the cerebellum of rats concerning the effects of secretin as a retrograde messenger, which enabled an easier GABA-release from the corn cells using a presynaptic, cAMP-dependent mechanism. This effect is described as relatively long-term (>30 min.). Owing to the fact that secretin receptors were not just found on the corn cells, but also on the Purkinje cells, it would be plausible to suppose, in addition, the existence of an autocrine component in the regulation of this synapse. It is possible that Purkinje cells stabilize their resting potential following a successful depolarization by means of an increase in the inhibitory afferences of the corn cells. Should a similar model be valid in accordance with the hippocampus, for example between basket cells and pyramidal cells, this would explain the GABA-increase measured in this study.

Electrophysiological examinations conducted by Yang *et al.* (2004a) in the nucleus tractus solitarius of the rat described a different neuro-modulatory effect of secretin, that is, a depolarizing one, not blockable by tetrodotoxin. It is possible that not only secretin of neuronal origin is involved in central nervous

regulations, but also secretin of other tissue origins, especially of the gastrointestinal tract. Studies conducted by Banks *et al.* (2002) demonstrate the ability for secretin as well as for secretin analogon ^{131}I-secretin to pass by the blood brain barrier. They investigated the penetration of a radioactive marked ^{131}I-secretin-analogon through the blood brain barrier in 4-week-old mice. The substance passed through the blood brain barrier as an intact molecule and broke through into all brain regions, especially however into the hypothalamus and the cerebrospinal fluid. Banks *et al.* postulated that the secretin analogon passes through the plexus choroideus through a satiable transport process, and passes the vascular blood brain barrier through transmembrane diffusion.

As with most peptides secretin possesses a comparatively short half-life in blood circulation owing to degradation and inactivation. Similar to the amphiphatic molecule VIP and PACAP, which stems from the same protein family, it can however self-assemble into micells in liquid solution and interacts with biomimetic phospholipid membranes, in which the secretin molecules experience a conformation change; it is possible that these physical properties distinctly increase its bioactivity as a pharmacon, yet perhaps already in fact in endogen concentrations (Gandhi *et al.*, 2002; Krishnadas *et al.*, 2003).

The i.c.v. injection of secretin as well as an i.p.-application of secretin led to an increase in concentration of glutamate and GABA in the hippocampus of the rat (Kuntz *et al.*, 2004). The main question whether the measured effect came about through central nervous, vagus mediated, or hormonal type effect is still unanswered. If the effect was due to central neuromodulation, there must have been imitation of central and vagal mediated secretin effects in relation to extracellular transmitter concentrations in the hippocampus; this would only be resolved through an intraperitoneal administration of secretin in vagotomized rats.

Studies by Yang *et al.* (2004) confirm the role of the nervus vagus on i.p. application of secretin through the observation of stronger Fos-expression in different areas of the CNS, even if not in the hippocampus itself. Fos-activation through i.c.v.-application of secretin however seems to have a more extensive central nervous activating spectrum than the administration i.p., and also activates cortical regions (Welch *et al.*, 2003).

The "brain-gut-peptide" secretin seems to be a part of a gastrointestinal-central nervous interaction: as previously mentioned, the central amygdale nucleus area can be activated by secretin in relation to Fos-expression; this is achieved through intraperitoneal administration via vagal afferences (Yang *et al.*, 2004b), through intravenous administration via unclear transmission (Goulet *et al.*, 2001), but, especially remarkable, also directly through i.c.v. administration (Welch *et al.*, 2003). A slightly elevated plasma secretin mirror ($\sim$0.029 μg/kg/h), that did not itself lead to any endocrine activation of the pancreas, was able in experiments on the rat to achieve a significant strengthening of the pancreas

secretion through electrical stimulation of the medial amygdale nucleus area (Jo *et al.*, 1994).

It has been proven that the central amygdala nucleus neurons connect to neurons of the vagal dorsal nucleus complex, and in fact to the sensible nucleus tractus solitarius, as well as to the nucleus dorsalis of the nervus vagus; these projections appear to be of a mainly restrictive character and modulate the vagal reflex of the gastrointestinal tract (Lyubashina, 2004; Zhang *et al.*, 2003).

A further indication that the dorsal vagal nucleus complex functions as the deciding relay station in the transmission of vagal stimulation to brain nuclei in the amygdala and hippocampus, is shown in a work completed by Marvel *et al.* (2004). The peripheral application of lipopolysaccharide, an immune activator that induces behavioral symptoms as well as social retreat, was able to be blocked on the level of vagal brain nuclei.

The amygdala is a brain structure whose altered function is seen as a present neuropathological finding in autistic illnesses; its function is considered as essential for the development of a "social brain." In studies using fMRI autistic patients showed, contrary to healthy control persons, no activation of the amygdala in an exercise where parts of the eyes were shown and the patients were to explain the feelings or thoughts being expressed (Baron-Cohen *et al.*, 2000). Since secretin can activate the amygdala, which can itself strengthen the secretinergic pancreas secretion, it is conceivable that a sufficient presence of secretin in the organism, that is, in the gastrointestinal tract, as well as in the CNS, enables for gastrointestinal and amygdala function; should this loop be broken up, exogenic secretin could help to normalize its function.

It is therefore conceivable that secretin could enable positive therapeutic effects only in a subtype of autism since autistic illness is of an extremely heterogenic nature and is associated with a wide spectrum of co-morbidities especially diarrhea, as some authors suggest. Following on from the first descriptions given by Horvath *et al.* (1998), there is also evidence in a study conducted by Kern *et al.* (2002) of the benefit of secretin injections in autistic children with active chronic diarrhea. D'Eufemia described a diarrhea illness observed in autistic people, which coincides with a change in the intestinal permeability (D'Eufemia *et al.*, 1996); there were also diminished amounts of digestive enzymes, sulfation capacity of the liver, and an increased pancreatic secretion in secretin stimulation tests (Horvath and Perman, 2002). Furlano *et al.* (2001) demonstrated histologically in the stomach of autistic children a damaged epithelial glycosaminoglycan composition, and described a lymphocytic colitis, which seemed to be different to other infectious stomach illnesses.

Secretin might in connection with pathophysiological situations of this type, by stimulation of a bicarbonate rich pancreas secretion or by an increase in the amygdala feed back, lead positively to a normalization of gastrointestinal and secondarily or primarily also to neuronal function. There are no descriptions of a lower secretin level in the blood of autistic persons. It is possible that this is a

group of children not suffering from gastrointestinal problems to any large extent and would therefore not profit from secretin. In a phase-II-study conducted by Repligen, in an attempt to identify a subgroup of autistic secretin responders, a better response was measured to a secretin injection for a group with calprotectin and chymotrypsin concentrations than for other groups involved in the study (www.repligen.com). There is as yet no published data on the evaluation and study design of the phase-III-study, which followed on from there.

An expression of secretin in the CNS was observed during an embryogenesis in mice (Siu *et al.*, 2005), which is an indication of a possible involvement of secretin in neuronal development. The genes coding for secretin and its receptor are not located in chromosomal areas that have been associated with autism. Several proteins involved in neuronal differentiation and synaptogenesis or in intracellular, not specific neuronal signal transduction path have been associated with autism. Likewise a possible candidate gene for an aetiological participation in autistic child development is a gene for the glutamatergic NMDA receptor (see for an overview IMGSAC, 2001). Furthermore there is evidence of disorders of the glutamatergic system in autism; an abnormally increased availability of the transcription product for the transport protein EAAT1 and for the glutamate receptor AMPA1 has been found, whilst at the same time the actual concentration of AMPA receptors in the cerebellum was abased (Purcell *et al.*, 2001). The function of the complex glutamatergic system of the CNS plays a central role in learning activities like phenomena of LTP and LTD in the hippocampus. Gwag and his colleagues demonstrated that an increase of the endogenous glutamate concentration in the dentate gyrus of rats through blockage of the glial metabolism caused a selective NMDA receptor induced increase of mRNA expression of nerve growth factor (NGF) in the corn cells. For a short time glutamate increase reached double the concentration of the basis level before falling back again within 3 h. This short signal was sufficient to trigger an increase in the transcription rate of the growth factor (Gwag *et al.*, 1997). Autism as an illness, which is associated with disturbed neurogenesis in the amygdala and the hippocampus among other affected brain areas (Aylward *et al.*, 1999), could be positively influenced by such growth stimuli. An increased concentration of serotonin was measured in plasma of autistic people, whereas concentrations of glutamine, glutamate, and GABA were demonstrated to be significantly reduced. Whilst a positive correlation between measured values for serotonin and GABA was found in normal people, these two parameters had a significantly negative correlation in autistic people (Rolf *et al.*, 1993). Dhossche *et al.* (2002) described elevated plasma GABA levels in autistic children aged between 5 and 15 years. Human platelets are classified as an established model, which reflects the monoamine—as well as amino acidic content of neurons.

In the neonatal, still developing hippocampus, GABA still functions as an excitatory brain transmitter, but not however in the adult brain (Ben Ari *et al.*,

1997); yet apart from this the neuronal GABAergic activity is an important signal for the synaptogenesis (Belhage *et al.*, 1998). Hussman (2001) formulated a hypothesis where a reduction of GABAergic inhibition exhibits a possible aetiological factor of autistic symptoms. In a biophysical model of the hippocampal CA3 region described by Wallenstein and Hasselmo (1997), learning and memory ability performance deteriorated as soon as the GABA receptor induced inhibitor was removed, as this caused a deterioration of quality sensory information through concurrent activity of intrinsic and afferent fibers. A decreased GABAergic inhibition could also lead to a glutamatergic hyperexcitation and subsequent damage to vulnerable target neurons, a mechanism considered to be relevant for the onset of diverse neurological illnesses. There is indeed evidence from metabolic studies using magnetic resonance spectroscopy (MRS), which shows an increased degradation of neuronal membranes in the dorsal prefrontal cortex of autistic people (Minshew *et al.*, 1993). In an autoradiographic study by Blatt *et al.* (2001) eight different receptors from four neurotransmitters (i.e., GABA, serotonin, acetylcholine, and glutamate) were marked with ligands in order to compare the quantity of their hippocampal expression in autistic and normal people. A significant decline in the high affinity binding to $GABA_A$-receptors was detected.

The stimulated release of GABA by secretin in the cerebellum (Ng *et al.*, 2002) may account for an improvement in learning processes in this part of the brain which typically exhibits anomalies in autistic people (Acosta, 2003; Fatemi *et al.*, 2002) and which is essential for the development of higher cognitive functions in child development (Allen and Courchesne, 2003). The long lasting character of modulation likewise affirms the possibility of such an effect.

In summary it seems conceivable that a strengthening of the glutamatergic and the GABAergic transmission could positively influence an existing deficient neuronal development. The connection at this level admittedly remains speculative, as long as a relevant clinically defined subgroup of autistic people cannot be identified, where the application of secretin yields a proven and replicable successful treatment. The Phase III Study by Repligen was prematurely aborted in January 2004, which means that a suitable study design is missing.

Recently a pilot study of 22 people appeared (Sheitman *et al.*, 2004) as well as a further clinical case description (Alamy *et al.*, 2004), where schizophrenic patients with autistic symptoms reacted to a single secretin injection with a clear but very quickly reversible reduction of their clinical symptoms. Whilst people in their immediate environment including their care attendants described these symptom improvements quite impressively, their evaluation is clearly difficult with respect to psychiatric documentation. It is also noteworthy that this deals with schizophrenic patients who in themselves exhibit difficult and therapy resistant characteristics. There are further studies being conducted by Repligen to evaluate the benefit of an application of secretin in schizophrenia and anxiety disorders (www.repligen.com).

Even if the glimmer of hope has dimmed that secretin is the long-awaited "wonder drug" in the treatment of autism, it is equally inappropriate to maintain that the initial changes in behavior described by the parents represent complete misinterpretations. There is now renewed interest in secretin as a neuropeptide. Research into the neuromodulatory potential of endogenous peptides is certainly an important aspect in the development of a more exact knowledge about the creation and therapy of psychiatric illnesses.

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PREDICTED ROLE OF SECRETIN AND OXYTOCIN IN THE TREATMENT OF BEHAVIORAL AND DEVELOPMENTAL DISORDERS: IMPLICATIONS FOR AUTISM

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The long-term goal of our work is to create a novel treatment for autism and to explain its pathogenesis. Based on a theory that views emotions and emotional behavior as stemming from dysregulations of a unified brain/gut network, we propose a new paradigm for the treatment of mental illness. This chapter reviews evidence that two neuropeptides, secretin and oxytocin, are critical in the conditioning of infant adaptive behavioral patterns and that peptidergic mechanisms are abnormal in developmental disorders such as autism.

Our clinical observations in the treatment of autism justify our laboratory investigations into the role of peptides in the neurological manifestations of visceral diseases encompassing emotional/visceral brain regions abnormal in

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autism. Importantly, our studies have thus far demonstrated that (1) visceral inflammation activates visceral/emotional brain regions in areas known to be abnormal in autism; (2) secretin, like oxytocin, activates many of the same visceral/emotional brain regions that are dysregulated in chronic cerebral and visceral disorders such as autism; (3) a structural basis for the mechanisms of action of secretin and oxytocin was clarified; (4) secretin as well as oxytocin is synthesized in the hypothalamus and may act on structures involved in the pathophysiology of autism; (5) secretin and oxytocin localize to perivascular and subependymal regions of the paraventricular hypothalamus, suggesting a chemosensory and secretory function.

We believe autism is the result of an adverse cascade of events that stems from one or more genetic/environmental insults. Over time, if uncompensated, the cascade leads to adverse conditioning of stress adaptation networks and results in various interrelated developmental psychological, neurological and immunological pathology, including autism.

Our laboratory is engaged in efforts to translate clinical experience in the treatment of autistic patients into bench findings. We believe that it is possible, regardless of etiology, to treat autism and related developmental disorders by intervening in stress response mechanisms with exogenous administration of peptide combinations.

I. Introduction

To date, there is no comprehensive treatment for the broad range of autistic symptomatology: seizures (Park, 2003); attentional/arousal dysregulation, attentional deficit hyperactive disorder (Booth *et al.*, 2003); obsessive-compulsive disorder (Hollander *et al.*, 2003); stereotypies (Militerni *et al.*, 2002); social isolation (Iqbal, 2002); attachment disorders (Kobayashi *et al.*, 2001; Tinbergen and Tinbergen, 1983); face recognition deficits (Ogai *et al.*, 2003; Schultz *et al.*, 2003); gaze aversions (Richer and Coss, 1976); gastrointestinal disorders (Gershon and D'Autreaux, 2003; Horvath and Perman, 2002; Horvath *et al.*, 1998; Torrente *et al.*, 2002); and altered heart rate variability (Corona *et al.*, 1998; Graveling and Brooke, 1978). Research in the field of autism and related disorders over the last twenty years has produced a large body of knowledge but has yet to produce any significant therapeutic outcomes. The search for even partially ameliorative interventions is a goal for all parents of autistic children.

Current pharmacologic treatments, such as anti-psychotics, mood stabilizers, antidepressants, anticonvulsants, and single peptides treat single symptoms, often with unacceptable side effects and/or limited therapeutic effects (Ansorge *et al.*, 2004; Coniglio *et al.*, 2001; Dunn-Geier *et al.*, 2000; Kern *et al.*, 2002; Lightdale

et al., 2001; Owley *et al.*, 2001; Posey and McDougle, 2000; Roberts *et al.*, 2001; Sandler, 1999). Psychotherapeutic measures have also been attempted with limited success (Diggle *et al.*, 2003; Langworthy-Lam *et al.*, 2002). Despite great efforts to understand the etiologies of autism and to devise treatments, autism and many severe behavioral disorders are still generally considered to be idiopathic and incurable.

The current paradigm for treating mental illness was greatly influenced by the work of Walter Cannon and Phillip Bard (Bard, 1928; Cannon, 1927). Their work set aside the visceral theory of emotions of William James and Carl Lange (James, 1884; Lange, 1885), who argued that emotional states of well- or ill-being are the result of visceral sensations (see Fig. 1A). Instead, Cannon and Bard argued that

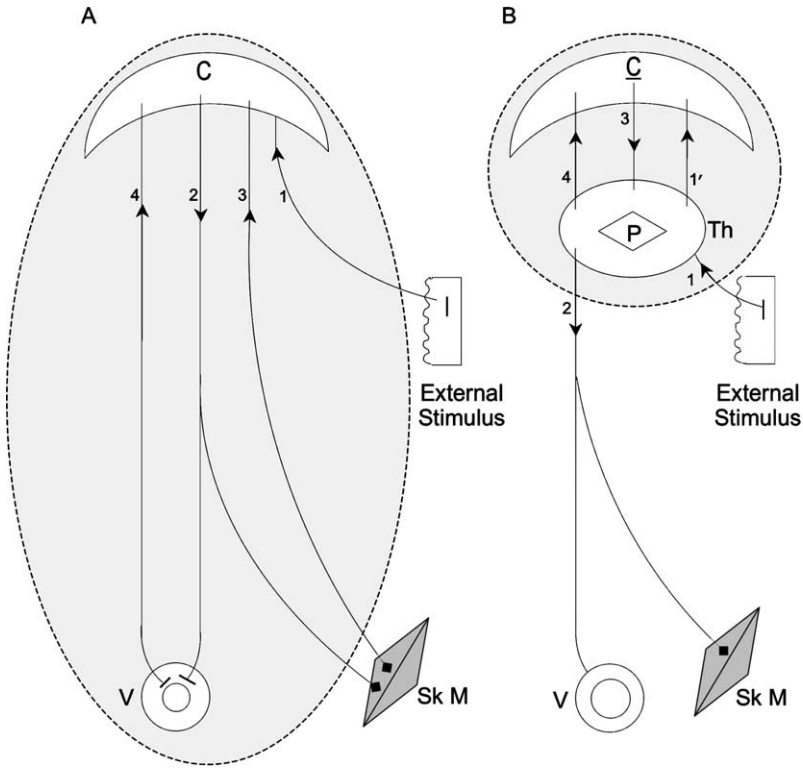

FIG. 1. Modified schematic of (A) James-Lange's visceral theory of emotion (1884–1885). (B) Cannon-Bard's revisionist thalamic theory of emotions (1927–1928) (Lissak and Molnar, 1975). According to James-Lange, emotions are the product of brain and gut, as indicated by bi-directional communication between viscera and brain and dotted line. Canon-Bard believed emotions are generated entirely in the brain, as indicated by one-way communication from brain to viscera and dotted line. **C** = Cortex; **P** = Pattern Generator; **Sk M** = Skeletomuscle; **Th** = Thalamus; **V** = Viscera.

emotional reactions do not stem from the viscera but result rather from behavioral patterns generated by the thalamus/hypothalamus (Fig. 1B). However, there has since been a growing acceptance that the viscera play a role in the generation of emotions (Damasio, 1994; LeDoux, 1998). Nonetheless, the scientific and health care communities still assume to a very large extent that behavior originates in the brain and therefore, in order to affect behavior, one must intervene in the processes of the brain. This chapter will present findings that support a revised theory on the origin and nature of behavior, one that logically calls for a new paradigm in the treatment of developmental and behavioral disorders.

We will argue that rather than originating in the brain, developmental disorders arise from dysregulation of a unified brain/gut system and are the result of a cascade of interrelated psychological, neurological, and immunological reactions to unmodulated stress (see Fig. 2). Further, we will argue that it is possible to ameliorate developmental and behavioral disorders, regardless of etiology, by intervening in stress mechanisms with treatments that target both the brain and periphery simultaneously.

Clinical observations in the psychiatric practice of Welch form the framework for the concepts reviewed in this chapter (Tinbergen and Tinbergen, 1983; Welch, 1983, 1988; Welch and Chaput, 1988; Welch *et al.*, 2004c, 2006). Two seemingly disparate groups of patients, consisting of maternally deprived orphans and autistic children, were treated for two shared symptom complexes: behavioral symptoms such as lack of direct eye contact, indiscriminate approaches toward strangers, inability to respond to normal maternal nurturing, and odd or restricted food preferences, and gastrointestinal (GI) symptoms such as gut motility abnormalities, discomfort, and diarrhea. Welch developed an intervention that employs intense nurturing as a means of conditioning stress adaptation responses. The intervention led to concurrent amelioration of both behavioral and gut symptoms. In many cases following the intervention, direct eye contact between mother and child ensued, the child was able to benefit from normal nurturing, adverse behaviors were dramatically reduced, and GI symptoms abated. At the end of the intervention, mothers who had previously experienced childbirth often described feeling as though they had just given birth. These collective bedside observations led to a theory that the two groups share a common dysregulation of underlying stress mechanisms.

Welch attributed the striking changes observed between mother and child in the therapy to the simultaneous release of natural endogenous peptides, especially the bonding peptide oxytocin (Uvnas-Moberg 1989; Welch *et al.*, 2004c, 2005, 2006). A serendipitous discovery involving secretin (Horvath *et al.*, 1998) provided an additional candidate for the hypothesized peptidergic mechanism, as well as further support for a brain/gut theory of developmental disorders. Secretin, given as a single dose probe of abnormal GI function in three autistic boys, resulted in improved eye contact and verbal communication. Welch reasoned that treatment with continuous exogenous combined secretin/oxytocin peptides

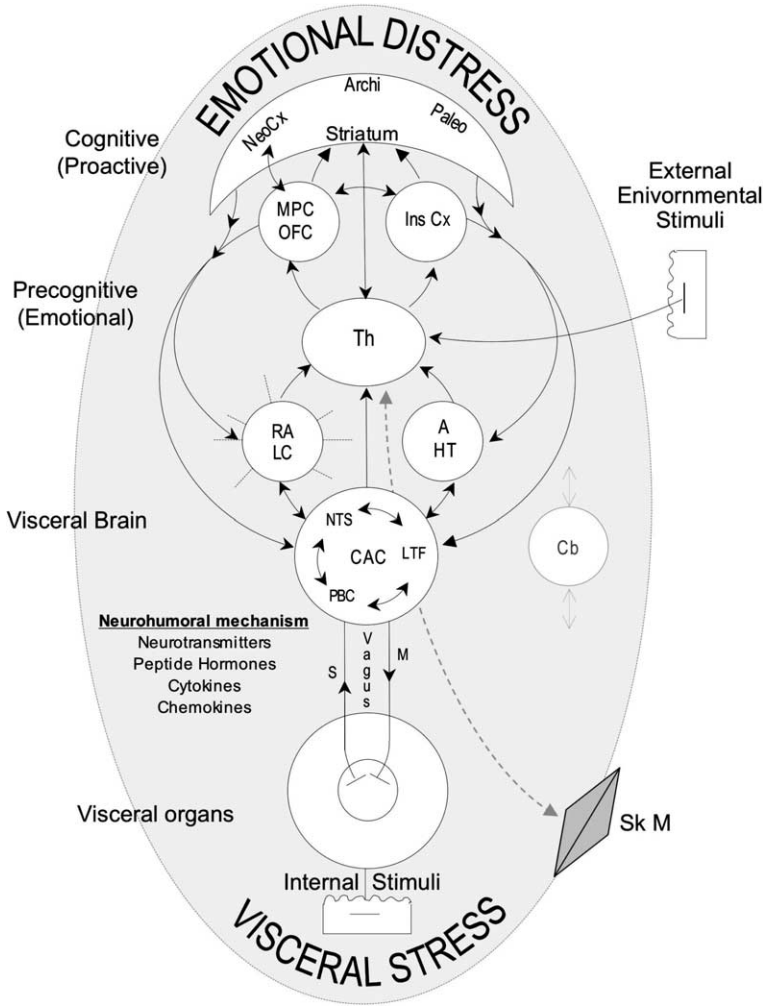

FIG. 2. Abbreviated schematic of the Welch-Ruggiero unified brain/gut theory of emotions. The simplified figure illustrates the fundamental circuits linking the viscera and emotional brain. The structures shown are sites of action of transmitters and peptides in mediating neurohumoral mechanisms sustaining adaptive behaviors accompanying arousal. Shaded area indicates viscera and brain are one system with bi-directional communication between the two, a theory that is in line with James-Lange (Fig. 1A). Most behavioral and pharmacologic therapies continue to target the brain and cognitive processes, assuming as did Cannon-Bard that emotions arise in the brain as a system separate from the viscera (Fig. 1B). **A** = Amygdala; **C** = Cortex; **CAC** = Central Autonomic Core; **Cb** = Cerebellum; **HP** = Hypothalamus; **Ins Cx** = Insular Cortex; **LTF** = Lateral Tegmental Field; **LC** = Locus Ceruleus; **M** = Motor; **MPC** = Medial Prefrontal Cortex; **NTS** = Nucleus of Solitary Tract; **OFC** = Orbital Frontal Cortex; **PBC** = Parabrachial Complex; **RA** = Raphe; **S** = Sensory; **Sk M** = Skeletal Muscle; **Th** = Thalamus; **V** = Viscera.

might replicate the physiological conditions that are elicited in normal reciprocal mother-infant interactions and ameliorate behavioral and GI symptoms in autism (Welch 1983, 1998, 2003b, 2005).

The relationship between brain/gut stress and developmental disorders such as autism is the framework for our scientific investigations. Prefrontal perceptual encoding mechanisms that regulate HPA stress axis and autonomic output to the viscera and immune system are impaired by gestational stress (Berger *et al.*, 2002). It is well established that early environmental stressors can permanently alter perceptual, emotional, intellectual, and social development; in autistic children, all of these are impaired. (Dawson *et al.*, 1998a,b). Autism is associated with high rates of visceral and immune disorders (Gupta *et al.*, 1998, 2000; White *et al.*, 2003) and familial autoimmunity is a risk factor (Szatmari, 1999).

We hypothesize that autism and associated disorders are the result of an adverse cascade of psychoneuroimmunological events that derive from one or more gene/environmental insults or unmitigated stressors. We also hypothesize that early intervention can interrupt the adverse cascade of events, thereby compensating for such insults and averting the further on-going sequelae that lead to severe chronic developmental disorders. Environmental insults can occur in utero or postnatally. Without intervention, the infant's stress profile will result in a failure to activate specific developmental programs, such as glucocorticoid and GABA receptor compositions. The cascade leads to a disruption in the stress-regulatory system of the developing infant, impairing ability to benefit from the caregiver's nurturing or stress-modulation. This disruption results in an interruption of key genetic developmental programs that are normally activated by peptidergic mechanisms. In the face of genetic/environmental stressors, the excess demands on the infant's stress-regulatory mechanisms make peptide modulation critical.

The failure we further hypothesize, persists until the peptide balance is restored. The earlier the silenced or arrested gene programs are activated by successful intervention, the less stress-induced damage will occur. Conversely, the longer the infant is unable to receive stress modulation, the more the infant's adaptation to environmental and emotional challenges is adversely conditioned. In such cases, in the face of unremitting environmental and emotional challenge, the infant adopts various maladaptive defense strategies that result in regressive adverse behaviors and a range of pathology. In the case of autistic children adverse behaviors include stereotypical movements, approach/avoidance behaviors, obsessiveness, compulsiveness, and tantrums.

We will report findings from our laboratory and we will review findings on transmitters and peptides that have been found to be dysregulated in developmental disorders, including peptidergic mechanisms that reset biological systems perturbed pre- and/or postnatally by unmodulated stress. We will examine the role that hypothalamic/gut peptides play in the modulation of these systems, especially as occurs naturally in the process of maternal nurturing. Finally, we will discuss the hypothesis that peptidergic mechanisms may lead to new clinical and

pharmacologic therapies, and chart a future course for research and treatment of autism and related disorders.

II. Background

A. GABA, Genes, Environment, and Peptides

The fact that GABA inhibitory transmitter systems are genetically altered in autism (Lamb *et al.*, 2002; Ma *et al.*, 2005) is complicated by the fact that GABA receptor genes could also be activated or silenced environmentally (Caldji *et al.*, 2003, 2004). These findings may corroborate our clinical observations. Welch observed that autistic infants who were unable to engage in reciprocal bonding behaviors in response to maternal cues showed dramatic physiological change following successful treatment with intense maternal/infant nurturing (Tinbergen and Tinbergen, 1983; Welch, 1983, 1988; Welch and Chaput, 1988; Welch *et al.*, 2004c, 2006). Hofer may have identified the same phenomenon when he referred to "hidden regulators" of the mother/infant interaction (Hofer, 1994). Recent genetic research using animal models may offer a more compelling explanation of the phenomenon in terms of powerful cellular mechanisms underlying maternal/infant interactions.

Environmental effects can determine the activation status of a gene. It is common to think of gene effects as fixed. In fact, genes are activated or silenced continuously. Therefore, when environmental events silence or fail to activate a gene program, the outcome can be as deleterious as a genetic defect or abnormality, such as the $GABA_A$ receptor gene abnormality on Chromosome 15q11 associated with autism (Menold *et al.*, 2001).

GABA gene/environment interactions found in low-nurture rearing environments may be pertinent to children who do not or cannot benefit from normal maternal nurturing, such as in orphans and autistic children with abnormal face recognition and sensory processing. In animals, the level of maternal nurture that the offspring receives determines which gene programs for GABA receptor subunits are activated. The level of maternal care can permanently alter subunit composition of the $GABA_A$ receptor complex in brain regions that regulate responses to stress, including the amygdala. However, cross-fostering animal offspring of low-nurture mothers to high-nurture mothers reverses the subunit composition by selectively producing specific GABA receptor subunits (Caldji *et al.*, 2003). This finding may explain Welch's clinical observations that intense components of maternal nurture can ameliorate behavioral symptoms of low-nurture orphans.

In another experiment, cross-fostering of low-nurture animals to high-nurture mothers raised the numbers of glucocorticoid receptors in areas of hippocampus that determine stress reactivity (Meaney, 2004; Meaney and Szyf, 2005; Weaver

et al., 2004). Reduced numbers of glucocorticoid receptors were found in the brains of subjects with depression, bipolar disorder, and schizophrenia (Webster *et al.*, 2002). If this finding occurs in autism as well, it would lend further support to the concept that high nurture interventions might raise the numbers of hippocampal glucocorticoid receptors in autism.

GABA is a major inhibitory neurotransmitter responsible for sensory gating of stress-related information that influences behavioral, endocrine, and autonomic networks. It is implicated in many psychiatric disorders (Kalia, 2005; Lewis *et al.*, 2005; Roy-Byrne, 2005). Secretin and oxytocin facilitate GABA inhibition (Kuntz *et al.*, 2004; Zaninetti and Raggenbass, 2000). Secretin and oxytocin are abnormal in autism (Gershon and D'Autreaux, 2003; Green *et al.*, 2001). In rat hippocampus, an oxytocin agonist facilitates inhibitory transmission by exerting an excitatory action on the soma or dendrites of GABAergic interneurons (Zaninetti and Raggenbass, 2000), and systemic injections of secretin increase concentrations of GABA (Kuntz *et al.*, 2004). Oxytocin and/or secretin deficits in autism could further complicate genetic GABA abnormalities. If so, it is possible that endogenous up-regulation via intense nurture or exogenous secretin and/or oxytocin could compensate for genetic GABA abnormalities in autistic children by triggering key developmental gene programs.

In addition to these abnormal glucocorticoid receptor findings in subjects with severe mental illness, Knable and colleagues found hippocampal abnormalities of reelin and brain-derived neurotrophic factor (BDNF) (Knable *et al.*, 2004). A genetic abnormality of reelin has been identified in autism (Persico *et al.*, 2001), and blood levels of BDNF were abnormal in a cohort of neonates later diagnosed with autism (Nelson *et al.*, 2001). The reelin findings suggest a dysfunction of inhibitory GABAergic interneurons (Knable *et al.*, 2004).

The reason autistic children do not respond to normal maternal cues is poorly understood. However, one possible factor could be central deficits that occur in sensory processing and face recognition. Whatever the reason, we hypothesize that since autistic children are unable to recognize or respond to normal maternal nurturing, they develop the genetic profile of low-nurture animals (with low glucocorticoid and altered GABA receptor composition). If this is the case, it may be possible to mimic the conditions of a high-nurture internal environment in autistic children through early up-regulation of endogenous peptides or early administration of exogenous peptides. In this way, children diagnosed with autistic symptoms at an early age and treated with peptides may be capable of accepting and reciprocating their mothers' nurturing. An important question is the extent to which exogenous peptides might overcome abnormalities of peptides, GABA, glucocorticoid receptors, cytokines, reelin, and other molecules in the stress cascade. If key developmental gene programs are activated, the cascade of adverse stress adaptation conditioning may be averted, thereby preventing the ongoing damage of unremitting stress in autism.

B. Secretin's Role in Regulating Stress

The role of secretin in maintaining homeostasis has long been established: anti-stress gastric hormonal action (Bayliss and Starling, 1902), deacidification of the gut (Jin *et al.*, 1994), stimulation of hepatic bile flow (McGill *et al.*, 1994), increase of coronary blood flow (Gunnes *et al.*, 1983, 1985), and increased lipolysis during fasting and muscular exercise (Bell *et al.*, 1984). In an animal, secretin is synthesized by the pancreas and colon (Lopez *et al.*, 1995) and by flora that inhabit the gut (Gauthier *et al.*, 2003).

Secretin's role as a peripheral stress-regulatory hormone and central neuromodulator of stress-adaptation responses has been suggested by earlier structural and functional studies (Chang *et al.*, 1985; Charleton *et al.*, 1981; Fuxe *et al.*, 1979; Itoh *et al.*, 1991; Mutt *et al.*, 1979; O'Donohue *et al.*, 1981; Samson *et al.*, 1984). Secretin-releasing peptide and secretin are secreted as part of unified, vagally-mediated behavioral and reflex response patterns (Chey and Chang, 2001). These peptides are triggered by stress-related increases in gastrin and gastric acid output in the gut (Li *et al.*, 1998). The prefrontal cortex and subcortical outlets of emotional memory are then conditioned to increase sympathetic output to the GI tract when stressed. The prefrontal perceptual encoding autonomic control mechanism modulates sympathetic/vagal discharges and visceral activity patterns (Ruggiero *et al.*, 1993, 1998). Stress-related increase in hydrochloric acid results in hyper-drive of the dorsal motor vagal output to gastric parietal cells, requiring compensatory increases in secretin cell output. The long-term impact of sustained stress is a dysregulation of finely tuned vagal reflex networks. It is possible that the decreased number of secretin cells (50% fewer) reported in autistic guts (Gershon and D'Autreaux, 2003) may be the result of unremitting stress-induced dysregulation that eventually leads to cell arrest or apoptosis of secretinergic cells. Inadequate amounts of secretin or inability to up-regulate secretin in the face of stress could make the infant vulnerable to GI pathology.

Secretin has been found to peripherally and centrally activate the dorsal vagal complex in the brain via the vagus and spinal nerves (Westlund *et al.*, 1996). The prefrontal cortical perceptual encoding areas (OFC, Ins Cx,) and subcortical outlets of emotional expression (Th, A, HP) (Fig. 2) are impaired by prenatal stress (Fumagalli *et al.*, 2004). This network converges on the dorsal vagal complex, impairing the parasympathetic vagal discharges to the viscera (Ruggiero *et al.*, 1993). Normally, activation of the nucleus of the solitary tract (NTS) within the dorsal vagal complex helps maintain homeostasis through modulation of behavioral, autonomic, and endocrine systems (Williams *et al.*, 2001). This dorsal vagal complex reflex response pattern is viscerally conditioned by components of maternal nurturing, such as breastfeeding and vocalization, which condition the infant to alternate between swallowing and breathing and, later in development, to alternate between talking and listening (Porges, 1995). In

their vagal circuit of emotion regulation theory, Porges *et al.* propose that cardiac vagal tone is of such importance that it can serve as an index of emotion regulation (Porges *et al.*, 1994).

It is important to note that Cannon's main criticism of William James's visceral theory of emotions (James, 1884) (Fig. 1A) was based on evidence showing that "total" separation of the viscera from the central nervous system (i.e., total destruction of the sympathetic and spinal sensory roots) does not alter emotional behavior. Cannon offered this as proof that emotions must arise in the brain (Cannon, 1927) (Fig. 1B). Though he was an expert on the autonomic nervous system, a devotee of Darwin, a lifetime friend of Pavlov, and a firm believer in the role of stress adaptation conditioning in the maintenance of homeostasis, Cannon, and until recently most others, overlooked the vagal brain/gut pathway and the profound role that the vagus nerve plays in controlling and conditioning behavior and emotions. We theorize that the vagus nerve serves as a primary pathway between the brain and gut by which peptides such as secretin and oxytocin influence dysregulated stress response patterns such as occur in autism (Fig. 2).

Secretin is secreted in response to breastfeeding (Zabielski *et al.*, 1994). In an animal model, the colostrum content of breast milk during the immediate post-partum period is a greater stimulus to secretin release than the milk itself (Guilloteau *et al.*, 1992). Studies on infantile autism reveal a lower incidence of breastfeeding (Tanoue and Oda, 1989). Further study of the relationship between low or absent breastfeeding and autism could reveal whether replacement of key stress-modulating peptides such as secretin and oxytocin could help offset the deficit.

Secretin levels are elevated in diseases such as cystic fibrosis, hyaline membrane disease (Boccia *et al.*, 2001), and Crohn's disease (Teufel *et al.*, 1986), all of which are associated with GI abnormalities. Interestingly, vasoactive intestinal peptide (VIP), another member of the secretin family, was elevated in neonates later diagnosed as autistic, as compared to children with normal development (Nelson *et al.*, 2001). Human neonates exhibit excessive gastric acid and secretin output, as assayed in two-day olds, whereas secretin expression reaches the mature pattern by the second postnatal week in healthy pre-term infants (Lucas *et al.*, 1980a, b). Sick infants with hyaline membrane disease exhibit sustained up-regulation of secretin secondary to both starvation (Lucas *et al.*, 1980a, b) and respiratory stress analogous to that induced by colchicine (Jones and Gonzalez-Lima, 2001). These data suggest that secretin is up-regulated in the periphery in response to stress.

The central actions of secretin are less clear. Several studies have reported secretin in widespread areas of the central nervous system (Chang *et al.*, 1985; Mutt *et al.*, 1979; O'Donohue *et al.*, 1981), including the hypothalamus (Chang *et al.*, 1985; Charlton *et al.*, 1981; Mutt *et al.*, 1979; Samson *et al.*, 1984). Others

reported expression of an mRNA secretin precursor in the brainstem, thalamus, and cerebral cortex, as well as in the hypothalamus (Itoh *et al.*, 1991; Ohta *et al.*, 1992). Several studies suggest that secretin is synthesized endogenously in the central nervous system (Fuxe *et al.*, 1979; Itoh *et al.*, 1991; O'Donohue *et al.*, 1981; Ohta *et al.*, 1992). Earlier studies have localized secretin and its presumptive receptor binding sites to viscerolimbic brain regions involved in central autonomic regulation (Itoh *et al.*, 1991; Nozaki *et al.*, 2002; Ohta *et al.*, 1992). The precise location of the secretinergic cells was not established by any of the above techniques, which lacked the single-cell resolution of our immunocytochemical methods (Welch *et al.*, 2004a).

Abnormalities of the cerebellum could explain a dysfunction of its role in emotional memory, learning of motor skills, and autonomic controls in autism. Cerebellar vermal connections with the hippocampal formation, amygdala, and hypothalamus form an integrated network implicated in adverse conditioning of fear responses (Sacchetti *et al.*, 2005). Immunohistochemical techniques used to localize secretin have shown the highest immunoreactivity in the Purkinje cells of the cerebellum (Koves *et al.*, 2002). Reduced numbers and volume of Purkinje cells have been reported in the cerebellum of autistic patients (Bailey *et al.*, 1998; Bauman and Kemper, 2005). The cerebellum is very important in development of conditioned behaviors. It is the great cerebral ganglion, receiving mental and physical information and transmitting both to the thalamocortical/striatal networks and to the viscera and musculoskeletal systems (Carpenter, 1996). These circuits are highly modified by postnatal experience. Inasmuch as secretin facilitates GABA transmission in the cerebellum (Yung *et al.*, 2001), it is possible that secretin administration could have a beneficial effect on cerebellar neural transmission in autism.

Behavioral changes that follow injection of secretin into the cerebroventricular system in rats include significantly increased defecation, altered respiration, and decreased novel-object approaches and open-field locomotor activity (Charlton *et al.*, 1981). Banks determined that secretin could cross the blood-brain barrier in mice injected with a radiolabeled secretin analogue (Banks *et al.*, 2002). The compound was reported to have entered every brain region, with the fastest uptake in the hypothalamus and the hippocampus, two brain regions that exhibit developmental abnormalities in autistic patients (Bauman and Kemper, 1985; Haznedar *et al.*, 2000; Ogai *et al.*, 2003; Schultz *et al.*, 2003). In another study, secretin was found to bind with specificity and high affinity to receptors in the nucleus of the solitary tract, thalamus, hypothalamus, and cerebral cortex (Nozaki *et al.*, 2002), all of which are sites of pathology in autism.

Secretin may influence behavior by peripheral and central mechanisms that protect against visceral stressors. Both experimental and clinical data previously cited raise the possibility that neuro-psychiatric and functional GI abnormalities in autism could be secondary to hypoxia, a known environmental insult in

developmental disorders (Davis *et al.*, 1992). Secretin receptors as well as oxytocin receptors couple to G-proteins. G-proteins stimulate adenylate cyclase, which leads to the production of cyclic adenosine monophosphate (cAMP) (Harmar, 2001). In gut epithelial cells impaired by hypoxia in their ability to generate cAMP, pharmacologic elevation of cAMP normalizes both polymorphonuclear-induced permeability changes and restoration of barrier function (Friedman *et al.*, 1998). Such permeability changes have been found in autistic children (D'Eufemia *et al.*, 1996). Secretin is known to elevate cAMP (Fremeau *et al.*, 1986), and has been reported to decrease intestinal permeability in 13 of 20 autistic children (Horvath and Perman, 2002). We hypothesize that synthesis and secretion of secretin on demand may ameliorate hypoxia and other metabolic challenges of perinatal stress via two known mechanisms of action: as a vasodilator (Gandhi *et al.*, 2002), and as a gastric protective hormone (Bayliss and Starling, 1902). Various studies have illuminated responses to hypoxia by neurotransmitter and neuropeptide systems in the cardiovascular and GI control regions of the medulla oblongata (Iadecola *et al.*, 1993; Ruggiero *et al.*, 1993, 1998; Talman and Kelkar, 1993). Taken together the data suggest that systematic studies should be performed to investigate hypoxic changes in autism.

C. Oxytocin's Role in Regulating Stress

In an experimental model, Uvnas-Moberg showed that oxytocin is an antistress hormone, decreasing blood pressure and offsetting flight-or-fight and corticotropin-releasing-hormone (CRH) and norepinephrine responses to stressors (Uvnas-Moberg, 1997). Oxytocin was administered to rats I.C.V. for a 5-day period, resulting in sedation, diminished blood pressure, decreased corticosterone levels, and increased release of vagally controlled hormones. The fact that these effects were long-lasting, persisted for several weeks, and were not reversed by oxytocin antagonists, indicated to her that "secondary mechanisms" had been activated. While these secondary mechanisms have not been identified, it is possible that they involve the gene program activation in Caldj's experiments cited in the previous section (Caldji *et al.*, 2003; Weaver *et al.*, 2004). In any case, it is clear that oxytocin has a powerful role in regulating stress.

As central oxytocin pathways develop postnatally, they mature in their ability to influence vagally mediated digestive functions. Paraventricular hypothalamic oxytocinergic neurons already present from birth modulate vagal digestive motor functions via projections to the nucleus of the solitary tract and dorsal motor nucleus of the vagus in rats (McCann and Rogers, 1990). The cumulative length of the fibers increases between 23-fold and 94-fold between birth and adulthood (Rinaman, 1998). Oxytocin activates parietal cells that secrete acid into the lumen of the stomach. Secretin is then secreted into the bloodstream in

response to acid in the stomach. Oxytocin also stimulates cholecystokinin, with which it is co-localized in the brain. Cholecystokinin stimulates bile excretion, slows gastric emptying, and makes the mother and child sleepy (Uvnas-Moberg, 1989).

Oxytocin affects the gut through regulation of vascular tone (Jankowski *et al.*, 2000). Oxytocin acts on vagal neurons via voltage gated current, which is sodium dependent and modulated by calcium (Raggenbass and Dreifuss, 1992). Oxytocin is delivered by a humoral route and by direct innervation (Sofroniew *et al.*, 1981). The humoral route involves the production of oxytocin in the hypothalamus which projects fibers to the pituitary, from which oxytocin is secreted into the circulation.

Oxytocin release is stimulated by the act of breastfeeding, which in turn stimulates lactation (Hatton *et al.*, 1992; Matthiesen, 2001; Pedersen and Boccia, 2002). Breastfeeding is a major source of vagal stimulation (Uvnas-Moberg, 1989). In infants, vagal function can have profound influences on development. Breastfeeding confers long-term protection against GI inflammation (Barlow *et al.*, 1974). Somatostatin, a polypeptide hormone produced chiefly by the hypothalamus that inhibits the secretion of various other hormones, such as somatotropin, glucagon, insulin, thyrotropin, and gastrin, is inhibited during periods of vagal stimulation (Eriksson *et al.*, 1994). When continuous nursing is absent or low, vagal stimulation is low. Consequently, levels of oxytocin, VIP, prolactin, and other gut hormones such as secretin are decreased and the level of somatostatin is increased. Uvnas-Moberg has demonstrated that somatostatin (1) is secreted by a broad range of tissues, including pancreas, intestinal tract, and regions of the central nervous system; (2) inhibits gut function and is increased in level 10-fold during sickness, resulting in decreased motility, decreased HCl output, and decreased discharge of bile; and (3) blocks nutrient absorption, release of gastrin and cholecystokinin, and their growth-promoting effects (Uvnas-Moberg, 1989). We hypothesize that exogenous secretin/oxytocin peptide therapy could compensate for the effects of increased somatostatin in sick children.

Perhaps most important, suckling by the infant triggers a vagally-induced gut hormone response in the infant as well as in the mother, synchronizing their metabolism and inducing mild sedation or anxiolysis in the nursing pair (Uvnas-Moberg, 1987). The mechanisms by which this interaction occurs are in part related to vagal control of cardiac reactivity found in nursing women. Release of oxytocin influences the physiological state of the mother and her mothering patterns, increasing parasympathetic and decreasing sympathetic tone in the case of normal nurturing (Uvnas-Moberg, 1989). Mothering patterns and the mother's physiological state in turn influence the child's physiological state and behavior (Francis *et al.*, 2002). Indeed, non-nursing mothers experience increased sympathetic and decreased parasympathetic tone (Altemus *et al.*, 2001). Our clinical observation of mothers of adopted children with non-nursing histories

reflects this finding. The mothers are anxious and hypervigilant with the children. We also observe that adopted children with non-nursing histories demonstrate symptoms of sympathetic imbalance, including anxiety, hyperactivity, and hypervigilance. Furthermore, after therapy that includes components of intense maternal nurturing, the sympathetic balance (e.g., hypothesized peptidergic balance) of both the adoptive mother and the adopted child is restored. This outcome suggests that it may be possible to intervene in the peptidergic mechanisms of the mother and child to overcome maladaptive stress response patterns, regardless of etiology, in order to restore homeostasis.

Homeostasis is in part maintained by hormonal priming, which normally occurs during pregnancy. Once the mother begins to interact with her offspring, purely endogenous central release of neuropeptides and neurotransmitters decreases her anxiety. In animal mothers, the beneficial effects of hormonal priming cannot continue without mother-pup interaction. Either $GABA_A$ receptor antagonism or oxytocin receptor antagonism in caudal periaqueductal gray increases anxiety to levels typically found in virgin animals (Lonstein, 2005). In fact, without prior hormonal priming, virgin animals induced with maternal behavior by exposure to pups did not show any decrease in anxiety (Ferreira, 2002; Stern and MacKinnon, 1976).

Suckling initiates secretion of oxytocin from the hypothalamus and prolactin from the anterior pituitary. When the vagus is stimulated, such as during suckling, the levels of oxytocin, secretin, prolactin, and other GI hormones are increased. Suckling stimulates the vagus nerve of the mother and infant by way of sensory receptors on the nipple and in the mouth, respectively. The sensory receptors of the breast and mouth transmit via the parallel vagal and spinal pathways converging on the hypothalamus. The descending pathway in both mother and infant is from the vagal motor nucleus to the GI tract, pancreas, and other visceral organs including the heart (Uvnas-Moberg, 1989). The frequency and intensity of suckling stimulates peptidergic mechanisms and regulates cardiorespiratory and GI function in both mother and child, synchronizing their metabolism (Bornstein *et al.*, 2000).

The literature suggests that stressful conditions damage proteins such as peptides or inhibit their processing (Brostrom and Brostrom, 1998). In stressed infants, excess metabolic demands interfere with peptide function (Sanchez-Alvarez *et al.*, 2002). Oxytocin is a chemical antioxidant (Moosman and Behl, 2002). However, when peptides such as oxytocin undergo oxidation while scavenging for stress-induced free radicals, they lose their hormonal function (Ducrocq *et al.*, 1998). Furthermore, as oxytocin undergoes enzymatic conversion by brain stress responsive peptidases, oxytocin action in the brain may be compromised (Burbach *et al.*, 1980). We surmise that if oxytocin is compromised by stress, then it is likely that secretin is subject to similar stress effects.

Impaired peptidergic function due to excess metabolic demand at the cellular level can lead to adverse behavior. Oxytocin affects areas of social recognition and early environmental conditioning of stress adaptation patterns in animal models (Carter, 1998). Abnormally reared male rhesus monkeys with significant social deficits have persistently demonstrated reduced cerebrospinal fluid oxytocin levels and alterations in both CRH and vasopressin, suggesting that abnormal rearing impairs the development of brain systems critical to normal social and emotional competence. The autistic brain has been shown to have abnormal face recognition patterns (Dawson *et al.*, 2005). Autistic-like symptomatology in animals is associated with pathogenic rearing histories (Winslow, 2005). At the same time, there is evidence that restoration of oxytocin levels can ameliorate some of the adverse symptoms. Endogenous up-regulation in humans is concomitant with feelings of trust (Zak *et al.*, 2004), while exogenous up-regulation engenders feelings of trust (Kosfeld *et al.*, 2005). Oxytocin treatment fully restores social recognition in oxytocin mutant mice that fail to recognize familiar conspecifics (Ferguson *et al.*, 2001).

The fact that oxytocin receptors are distributed throughout the cerebellar behavioral and visceral reflex circuits (Vaccari *et al.*, 1998) provides additional insight into the functional neuroanatomical basis for therapeutic efficacy of oxytocin treatment in mediating stress adaptation patterns.

D. Secretin and Oxytocin's Interactions with Neurotransmitters

Secretin and oxytocin, acting on the brain and gut, likely subserve similar functions, such as the regulation of blood flow and metabolism (Gandhi *et al.*, 2002; Haraldsen *et al.*, 2002; Jankowski *et al.*, 2000). Oxytocin and its receptors are synthesized in the rat vasculature and may be involved in the regulation of vascular tone (Jankowski *et al.*, 2000). Oxytocin is known to modulate cerebral blood flow via a nitric oxide mechanism (Haraldsen *et al.*, 2002). Nitric oxide is a bi-product of stress, acting as an inhibitory neurotransmitter in the enteric nervous system (Kurjak *et al.*, 1996). Nitric oxide is an important biologic mediator in regulation of GI functions and plays a significant role in secretin-stimulated pancreatic secretion (Jyotheeswaran *et al.*, 2000). Nitric oxide at low concentrations functions as a signal in diverse physiological processes, including blood pressure control, neurotransmission, learning and memory, but at high concentrations nitric oxide functions as a defensive cytotoxin. Nitric oxide's absence or excess alters the cardiac effect of secretin in the same direction (Sitniewska *et al.*, 2000). Furthermore, nitric oxide inhibits oxytocin release (Engelmann and Ludwig, 2004; Kadekaro, 2004) and inhibits onset of maternal behavior in an animal model (Okere *et al.*, 1996).

Nitric oxide findings may have implications for the treatment of autistic children, some of whom have been found to have high plasma nitric oxide levels (Sweeten *et al.*, 2004). Nitric oxide has been implicated in the mechanism of tissue injury of inflammation in gastric mucosa. As stress-induced nitric oxide increases, glutathione levels decrease (Asanuma *et al.*, 2005). Plasma glutathione, another important antioxidant, is lower in autistic children (James *et al.*, 2004).

Decreased nitric oxide production in immune cells is also accompanied by increased IL-10 levels (Chang *et al.*, 2005). IL-10 (interleukin 10) is an anti-inflammatory cytokine that contributes to intestinal homeostasis (Maes *et al.*, 2003). Transgenic mice lacking IL-10 (IL-10-/-) spontaneously develop colitis (Annacker *et al.*, 2003). The interaction of secretin, oxytocin, and nitric oxide may be an indication that a combined secretin and oxytocin mechanism could prove beneficial in treating autism and inflammatory bowel disease (IBD). In duodenum of two autistic children, there were 50% fewer secretin (S) cells and 80% fewer (S) cells co-localizing serotonin (Gershon and D'Autreaux, 2003). Dysregulation of serotonin networks is found in IBD (Coates *et al.*, 2004) and in autistic children with familial hyperserotonemia (Cook, 1990; Leventhal *et al.*, 1990; Yirmiya *et al.*, 2001). Serotonin is proinflammatory to the gut (Linden *et al.*, 2003) and negatively regulates IFN gamma/IL-10 cytokine production ratios (Maes *et al.*, 2003). A recent study demonstrates altered IL-10 function in a population of autistic children with GI symptoms (Ashwood *et al.*, 2004). Findings relating secretin or oxytocin with serotonin and cytokines are important for our investigation of secretin/oxytocin treatment of the IL-10 mutant mouse model of IBD. Preliminary data show that combined secretin/oxytocin infusions ameliorate GI inflammation in the face of an IL-10 deficit (Welch *et al.*, 2003b).

Signaling molecules interact in the regulation of homeostasis and the stress response, notably via the paraventricular hypothalamus (Leong *et al.*, 2002), a site of oxytocinergic and secretinergic neurons (Windle *et al.*, 2004; Welch *et al.*, 2004b). Secretin and oxytocin interact with monoamines and angiotensin, which are abnormal in chronic cerebral and visceral disease (Chirguer *et al.*, 2001; Fuxe *et al.*, 1979; Jezova *et al.*, 2003; Li and Guyenet, 1996; Vacher *et al.*, 2002; Walker *et al.*, 1999). Hypothalamic oxytocin neurons modulate the activity patterns of brainstem structures synthesizing monoamines and regulating behavior, sleep, and arousal (Godino *et al.*, 2005). These interactions in turn subserve behavioral, endocrine, and autonomic regulation in response to homeostatic challenges. Normally, serotonin and norepinephrine control the expression of oxytocin in the endocrine hypothalamus and its secretion into the systemic circulation (Chriguer *et al.*, 2001; Vacher *et al.*, 2002). If secretin and oxytocin are dysregulated, it can be inferred that their role in reducing stress will be compromised.

Social isolation stress is a causal factor in stress ulcers and hippocampal pathology among insubordinate vervets (Uno *et al.*, 1989). The antagonizing of

angiotensin II type 1 (AT1) receptors restores modulation of HPA stress axis function (Armando *et al.*, 2001). It also prevents gastric mucosal injury (Bregonzio *et al.*, 2003). Insubordinate vervets and autistic children share non-compliance behaviors and social isolation (Breiner and Beck, 1984). The cingulate/hippocampal stress adaptation network is also a major site of pathology for both autistic children and vervets (Bauman and Kemper, 1985; Uno *et al.*, 1989). This network is implicated in the adverse conditioning of both GI functions (Gabry *et al.*, 2002; Uno *et al.*, 1989) and behavioral functions (Freeman *et al.*, 1997; Jones and Gonzalez-Lima, 2001). Secretin has been effective in ameliorating both gut and behavioral abnormalities of autistic children (Horvath and Perman, 2002; Horvath *et al.*, 1998; Lamson and Plaza, 2001; Kern *et al.*, 2004). Still to be investigated is whether secretin levels are altered by the social isolation of autistic children.

A particular interaction relevant to GI disorders is the relationship between angiotensin and secretin receptors. AT1 receptors are linked to social isolation stress (Armando *et al.*, 2001). AT1 receptors and secretin receptors are co-localized in endocytic vesicles (Walker *et al.*, 1999).

Taken together, these findings suggest that secretin, a vasodilator (Gandhi *et al.*, 2002), could be modulating the effect of AT1, a vasoconstrictor (Helou *et al.*, 2003). This modulation may take place, as Leong suggests, by "cross-talk" (interaction) of peptides at the level of the hypothalamus (Leong *et al.*, 2002) where angiotensin II attenuates GABAergic synaptic inputs (Li and Guyenet, 1996). If secretin is perhaps modulating effects of AT1 receptors via its effect on GABA, this interaction may explain why secretin ameliorates both GI symptoms (Armando *et al.*, 2001; Bregonzio *et al.*, 2003; Horvath and Perman, 2002; Uno *et al.*, 1989) and autistic behavioral symptoms (Horvath *et al.*, 1998; Kern *et al.*, 2004). Secretin and oxytocin have been shown to facilitate GABA inhibition in rat hippocampus (Kuntz *et al.*, 2004; Zaninetti and Raggenbass, 2000). If secretin and oxytocin both modulate GABA inhibition in the paraventricular hypothalamus, as secretin and oxytocin both do in the hippocampal formation, then a dual anti-stress action of oxytocin and secretin could predict ameliorative effects of combined secretin and oxytocin treatment in both GI and brain pathology at the level of the hippocampus and hypothalamus, as well as in the amygdala, the cerebellum, and in the periphery.

E. Brain/Gut Function

Since the gut and the brain develop from the same part of the human embryo, it is not surprising that the intestinal tract has such a rich nerve supply that it has been referred to as the "abdominal" or "second brain" (Gershon, 1998; Robinson, 1907). The gut shares many of the same types of neurons and

chemical transmitters with the brain, to which it is linked through the nucleus of the solitary tract. The nucleus of the solitary tract communicates visceral environmental information patterns to the emotional brain, triggering behavioral, endocrine, and autonomic stress adaptation patterns.

IBD is a GI disorder that activates the anterior olfactory nucleus, the piriform cortex, and the amygdala, providing specific support for a brain/gut connection in autism (Welch *et al.*, 2005). These brain areas are important in social recognition and early environmental conditioning of neonatal adaptive behaviors (Ferguson, *et al.*, 2001; Haxby *et al.*, 2002), both of which are deficient in autism (Dawson *et al.*, 1998; Ogai *et al.*, 2003; Pelphrey *et al.*, 2002; Winslow and Insel, 2002). A single injection of oxytocin has rescued the social recognition deficit of oxytocin mutant mice (Ferguson *et al.*, 2001).

There is evidence from both animal studies and human research that various peptides can ameliorate GI disorders. Oxytocin is known to protect infants against enterocolitis (Barlow *et al.*, 1974). Oxytocin also improves the antioxidative state of the colonic tissue and ameliorates oxidative colonic injury (Iseri *et al.*, 2005). VIP, a member of the secretin family of peptides, successfully treats IBD in an animal model (Abad *et al.*, 2003). In human research, epidermal growth factor enemas ameliorate IBD in humans (Sinha *et al.*, 2003). The actions of secretin may be additive and/or as important as oxytocin in brain/gut regulation. Secretin has long been known as a deacidification hormone that protects the gut (Bayliss and Starling, 1902). Gastroenterologist Horvath reported that systemic administration of secretin, given as a probe of abnormal GI function, resulted in improved behavior in autistic boys with abnormalities of gut function and decreased intestinal permeability in another group (Horvath and Perman, 2002; Horvath *et al.*, 1998).

Recent research points to the connection between behavioral and gut disorders. Early adverse events are associated with IBD (Ringel and Drossman, 2001). There is evidence of brain inflammation and gut pathology in autism (Vargas *et al.*, 2005; White, 2003). Visceral inflammation is a potent dysregulator of cognitive and emotional brain regions/states.

Both secretin and oxytocin have vasodilator functions (Gandhi *et al.*, 2002; Haraldsen *et al.*, 2002; Jankowski *et al.*, 2000). Secretin vasodilates the gut vasculature, as does VIP, another member of the secretin family of peptides (Furness *et al.*, 2004; Naruse *et al.*, 1998). The neuroprotective actions of VIP involve its ability to vasodilate cerebral blood vessels in the face of high stress (Dalsgaard *et al.*, 2003). Our detection of perivascular secretin- and oxytocin-like immunoreactivity (Fig. 3) provides evidence of their role in the regulation of cerebral blood flow similar to that played by VIP (Welch *et al.*, 2004b). These findings suggest that secretin and oxytocin influence cerebral perfusion and that both confer neuroprotection against hypoxia through peripheral visceral and cerebral vasodilation. The facts that cerebral perfusion deficits exist in autism

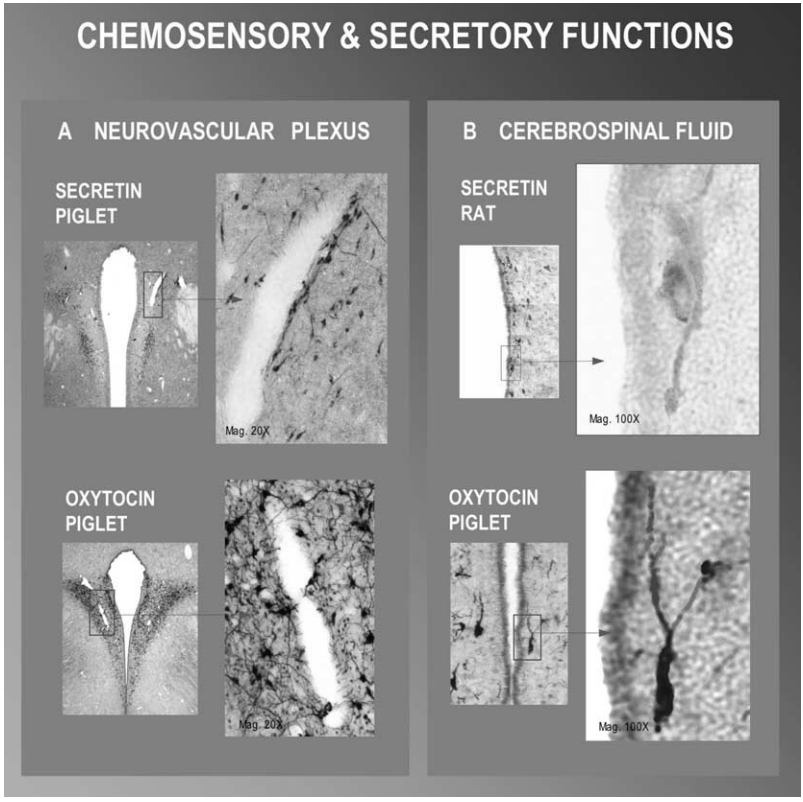

FIG. 3. Our studies showing secretin and oxytocin exchange across (A) the neurovascular plexus and (B) the CSF/ependymal interface of the third ventricle. Theoretically, neuropeptides may be secreted into or extracted from the CSF and blood stream. Secretin/oxytocin neurons may act as baro- and chemosensors, responding on demand to elevations of messenger molecules, stress hormones, and neurotransmitters. Secretin and oxytocin may be secreted into or extracted from the blood/CSF, where interactions may occur with classical neurotransmitter systems, such as GABA, serotonin, and dopamine, and with other stress regulatory peptides, such as corticotropin releasing hormone, vasopressin, and angiotensin (Welch *et al.*, 2004b).

(Haznedar *et al.*, 2000; Ohnishi *et al.*, 2000) and that oxytocin and secretin may be dysregulated in autism (Gershon and D'Autreaux, 2003; Green *et al.*, 2001) suggest that the two peptides may be connected in function and may share an oxidative stress mechanism.

To the best of our knowledge, no study has compared the cerebrovascular and brain activity patterns of autistic children and patients diagnosed with IBD. If the primary site of pathology in autism is an inflammatory/autoimmune condition involving the gut, then we would expect comparable patterns of brain

activation, altered transmitter/peptide receptor binding sites, and concomitant dysregulation of peripheral and cerebral blood flows in patients with either IBD or autism. It has been suggested that the neurologic complications of IBD involve an immune-mediated inflammatory process of cerebral vasculature (Dietrich and Erbguth, 2003; Lossos *et al.*, 1995). Both autism and IBD are associated with signs of immune dysregulation: major histocompatibility complex expression (Gupta, 2000; Matri *et al.*, 2003), familial predisposition to each (Szatmari, 1999; Cho, 2003), peptide dysregulations (Gershon and D'Autreaux, 2003; Green *et al.*, 2001; Kimura *et al.*, 1994; Kulman *et al.*, 2000; Teufel *et al.*, 1986), and altered cytokines (Singh, 1996; Gupta *et al.*, 1998; Dohi *et al.*, 2000).

Visceral stress is a dysregulator of cytokine/peptide interactions (Licinio *et al.*, 1999; Tannenbaum *et al.*, 2002). Such interactions are governed in complex ways in the brain via neural and humoral signaling pathways along the HPA axis and are critical to the neuro-immune-endocrine system (Haddad *et al.*, 2002). It is also known that chronic visceral stress is a potent dysregulator of cognitive/emotional brain regions (Traub *et al.*, 1996). It is conceivable, we think probable, that chronic inflammation compromises the brain's capacity to sense, synthesize, and react normally to inflammatory signals, thereby resulting in a range of neurologic and psychiatric pathology. If so, it would follow that visceral and emotional dysregulation should be viewed as inseparable and treated as a single dysregulation.

III. Findings

A. Central Hypotheses of Our Work

Our central hypotheses are as follows. The brain and body form a single physiological unit (Fig. 2). Neuropeptides and their transmitters act through neural-humoral pathways to influence the brain/gut simultaneously. Release or inhibition of neuropeptides determines physiologically regulated or dysregulated states and results in positive or negative stress adaptation conditioning. Physiologically dysregulated states may be ameliorated by neuropeptide therapy.

B. Secretin Activates Visceral Brain Regions That Are Abnormal in Autism

The aim of this study was to determine whether central networks are involved in the presumptive behavioral and autonomic regulatory actions of secretin (Welch *et al.*, 2003a), a gut hormone that has been reported to have ameliorative effects in autistic children.

Central neural responses monitored by regional c-*fos* gene expression were examined in response to intracerebroventricular secretin injection in awake, Sprague-Dawley male rats. Tissue sections were incubated in an antibody to the c-*fos* gene product, Fos, and processed immunohistochemically.

Secretin-infused rats showed altered numbers of Fos-immunoreactive nuclei, mainly in visceral and limbic areas of the brain. Secretin induced c-*fos* protein expression in the dorsal vagal complex, the area postrema, and its sub-postremal region of transition with the nucleus of the solitary tract and the commissural, medial parvicellular, and periventricular subnuclei. Secretin activated cells localized to the nucleus of the solitary tract projection fields: lateral reticular formation, locus ceruleus, ventral periaqueductal gray, and paraventricular thalamic nucleus, corresponding to the non-discriminative, stress-reactive, visceral thalamus. Based on these results and prior findings (Ruggiero *et al.*, 1998), we hypothesize that the visceral thalamus/cortical/striatal stress axis processes the drives (tensions) motivating expression of adaptive maternal/infant bonding behavior. In the hypothalamus, the predominant labeling mapped to the paraventricular hypothalamic nucleus, mainly its periventricular region and magnocellular subdivision. Secretin induced c-*fos* in the medial and central amygdala and the lateral septal complex. Dramatic secretin-activation of ependymal and subependymal nuclei lining the third ventricle contrasted with the absence of immunoreactivity in the age-matched controls.

Specific areas of the cerebral hemispheres were heavily labeled in the secretin-treated rats as compared to controls. The nuclear immunoreaction product was most heavily concentrated along the medial bank of the PFC, the orbitofrontal cortex, the anterior olfactory nucleus, and the piriform cortex. Relative to the Fos expression in untreated controls, secretin attenuated Fos immunoreactivity in the dorsal periaqueductal gray, the intralaminar thalamus, the lateral amygdala, the medial parvicellular hypothalamus, the somatosensory and association areas of the parietal cortex, and the motor cortex.

Significantly, secretin altered the activity of structures involved in behavioral conditioning of stress adaptation and visceral reflex reactions. Additionally, activation of third ventricular ependymal and subependymal cells provides a possible cellular mechanism for the behavioral regulatory actions. Secretin's behavioral effects in autistic children may involve these cellular mechanisms (Welch *et al.*, 2003a, 2004a, 2005).

Some of the brain areas that were either activated or attenuated in the secretin-treated rats overlap with areas known to be abnormal in the brains of autistic patients. Among the important areas where c-*fos* was decreased in the brains of rats treated with secretin was the medial parvicellular hypothalamus, which synthesizes the stress hormone releasing factor CRH.

Our findings in the rat suggest that secretin exerts a regulatory action on the brain, including stress adaptation networks and brain areas that influence the gut.

This new observation supports the idea that previously unsuccessful trials with secretin either included patients whose intestinal function was not appropriate for secretin treatment (Kern *et al.*, 2004), that the dosages and scheduling needed to be changed, or both.

Our study is the first systematic analysis of the actions of secretin in the brain of the laboratory rat using c-*fos* activation as a monitor of altered metabolism in various regions of interest. These areas are of interest because of their role in regulating behaviors related to stress adaptation and optimal function of visceral and immune organ systems. Data in this study predict that secretin may activate dysregulated behavioral and visceral regulatory circuits in autistic children.

C. Secretin Distribution and Specificity Support a Central Stress Neuroregulatory Role Applicable in Autism

We sought to determine whether secretin is synthesized centrally, specifically by the HPA axis, and to discuss secretin's possible neuroregulatory role in autism (Welch *et al.*, 2004a). Previous biochemical and radioimmunoassays demonstrated secretin immunoreactivity and high-affinity secretin receptor binding sites in forebrain regions (Charlton *et al.*, 1981; Mutt *et al.*, 1979). In our previous study, we demonstrated that secretin activated the same forebrain regions.

This study provided the first direct immunocytochemical demonstration of secretin immunoreactivity in the forebrain. It was the first to establish with single cell resolution that secretin is synthesized in the forebrain, specifically by the HPA stress axis. The specificity of our findings was demonstrated by preadsorption control data and by the fact that secretin and other members of its peptide family, PACAP, glucagon, and VIP, had different distributional patterns. For example, VIP staining cells were prominent in the suprachiasmatic nucleus which was virtually devoid of secretin.

Secretinergic neurons were heavily labeled in colchicine-treated rats, as compared to the untreated group which showed undetectable or light labeling. Secretin immunoreactivity was cytoplasmic and restricted to neurons of the anterior and middle regions of the hypothalamus and adjoining periventricular gray. Presumptive secretinergic neurons were concentrated in precise loci within the paraventricular/supraoptic and intercalated regions of the hypothalamus. Secretinergic cells were heavily concentrated and intensely stained in magnocellular, parvicellular, and periventricular divisions of the paraventricular nucleus.

Cells were localized to specific regions of the paraventricular and supraoptic hypothalamic nuclei, and to the ependyma and subependymal zone of the

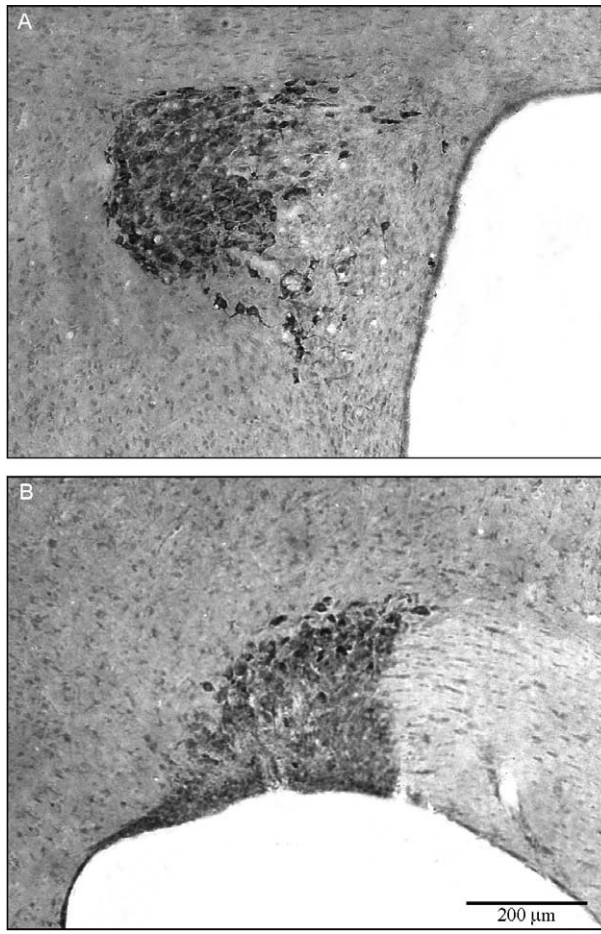

FIG. 4. Presumptive secretinergic neurons in the hypothalamic (A) paraventricular nucleus, and (B) supraoptic nucleus of a colchicine treated rat. This is the first direct immunocytochemical demonstration of secretin immunoreactivity in the forebrain. Control studies support specificity of secretin immunoreactivity. Dramatic differences in topographic distribution and density of secretinergic neurons from the distribution patterns of other members of the secretin/VIP/glucagon/PACAP family extend evidence of the existence of a secretinergic brain/gut stress-regulatory system (Welch *et al.*, 2004a).

third ventricle (see Fig. 4A). Secretinergic cells in the supraoptic nucleus were concentrated dorsally and extended medially, arching over the optic tract (Fig. 4B). Small numbers of cells were scattered among a heavily labeled neuropil in the ventral region of the supraoptic nucleus. A related mapping study established an

anatomical basis for potential interactions of secretin, oxytocin, and CRH in stress-responsive brain regions activated by visceral inflammation (Ruggiero *et al.*, 2005). The mapping study provided anatomical evidence that brain regions immunoreactive for S, OT, and/or CRH overlap. These regions are activated by stress and are common sites of pathology in cerebral and visceral diseases, including autism. Together with prior findings the results of the mapping study support the hypothesis that treatment with combined S and OT will counteract the stress effects of CRH in chronic visceral diseases with concomitant neurological manifestations.

The findings of our secretin distribution study offer evidence that the hypothalamus, like the gut, is capable of synthesizing secretin. The wide spectrum of behavioral, endocrine, and autonomic visceral effects of systemic peptide administration is consistent with this concept. A neuroregulatory relationship between the peripheral and central stress response systems is suggested, as is a dual peripheral/central role for secretin in conditioning both stress adaptation systems.

Secretin levels were up-regulated by colchicine, an exemplar of homeostatic stressors, compared to the low expression in untreated animals. Thus, secretin expression by brain and gut secretin cells is likely stress-related and, as suggested by the distribution patterns, may interact with other neuropeptides in conditioning stress-adaptation.

The fact that central colchicine up-regulated hypothalamic secretin expression suggests that secretin may be synthesized on demand in response to stress, a possible mechanism that may underlie secretin's role in autism. Secretinergic cells were found in comparable hypothalamic regions of the newborn pigs not treated with colchicine (Welch *et al.*, 2004b). This finding is consistent with evidence in human neonates expressing transient up-regulation of secretin in response to birth stressors (Lucas *et al.*, 1980a, b). Secretin is low constitutively and up-regulated by a physiological stressor in adult rats (Welch *et al.*, 2004a). Therefore, it is conceivable that secretin cells are depleted by stress in the same way that epinephrine cells are depleted in neurodegenerative diseases (Burke *et al.*, 2004). The fact that Gershon demonstrated 50% fewer S cells in the intestines of two autistic children than in controls (Gershon and D'Autreaux, 2003) suggests that autistic children with fewer secretin cells would not be able to up-regulate secretin in response to GI stressors, making them vulnerable to unremitting stress.

High-power optics revealed secretinergic periventricular neuronal processes contributing to subependymal perivascular fiber plexuses, which may serve as central chemosensors (see Fig. 3). Arrays of cells were diagonally organized in the nucleus intercalatus. In general, peripheral and central chemosensory receptors are activated by changes in chemical composition of the internal milieu, including a vast latticework of extracellular spaces referred to as Virchow-Robin spaces, which are abnormal in autism (Taber *et al.*, 2004). Chemosensors are activated by cerebral hypoxica/ischemic insults, hypercapnia, and chemical

stress factors triggering visceral/emotional stress reactions. Pathological physiological states result in altered permeabilities of cerebrospinal fluid (CSF) and blood brain barrier functions (Mark *et al.*, 2004). Secretin as well as oxytocin may be up-regulated on demand and released by terminals of hypothalamic, pituitary, bulbar, and spinal projections in response to such alterations in the chemical composition of this milieu. Furthermore, interactions of these peptides may occur with classical neurotransmitter systems, such as GABA, serotonin, and dopamine, as well as other stress regulatory peptides, such as CRH, vasopressin and angiotensin. Our findings extend previous studies characterizing central chemosensors that synthesize monoamines and peptides (Ruggiero *et al.*, 1985).

D. Brain Effects of Chronic IBD in Areas Abnormal in Autism and Treatment by Single Neuropeptides Secretin and Oxytocin

Recent research points to the connection between behavioral and gut disorders. In this study we sought to determine the extent to which chronic GI inflammation alters the functional activity of specific regions of the visceral and emotional brain in a rat model of acquired inflammatory bowel disease (IBD). Concomitantly, we tested the hypothesis that continuous infusion of stress regulatory peptides secretin or oxytocin will resolve gut inflammation and secondary neurological manifestations (Welch *et al.*, 2005).

Early adverse events are associated with IBD. In animal models, maternal deprivation and social isolation predispose to gastric erosion and brain pathology, as previously discussed. There is controversy over the effectiveness of secretin or oxytocin in the treatment of autism. The neurobiological basis of the efficacy of combined secretin/oxytocin treatment is supported by our preliminary findings in progress. This combined continuous neuropeptide therapy may ameliorate brain/gut dysregulation in autism.

IBD was induced in male Sprague-Dawley rats (n=10) with trinitrobenzene sulfonic acid (TNBS) vs. controls (n=11). IBD was characterized by moderate/severe infiltration of inflammatory cells. Secretin or oxytocin or equivolume saline was administered I.V. by Alzet pump for 20 days after disease onset.

IBD saline-, IBD secretin, and IBD oxytocin-treated animals had diarrhea and exhibited clearcut signs of sickness behaviors, lethargy, and lack of exploratory behavior. Qualitative analysis of the gut of TNBS treated animals showed substantial inflammatory infiltrates in the submucosa of the IBD colons (see Fig. 5A), whereas the guts of control animals demonstrated no inflammation (Fig. 5B). Qualitative analysis of the forebrain revealed c-*fos* induction in the rats with IBD: paleo, archi, meso, insular, and orbitofrontal cortices and subcortical regions

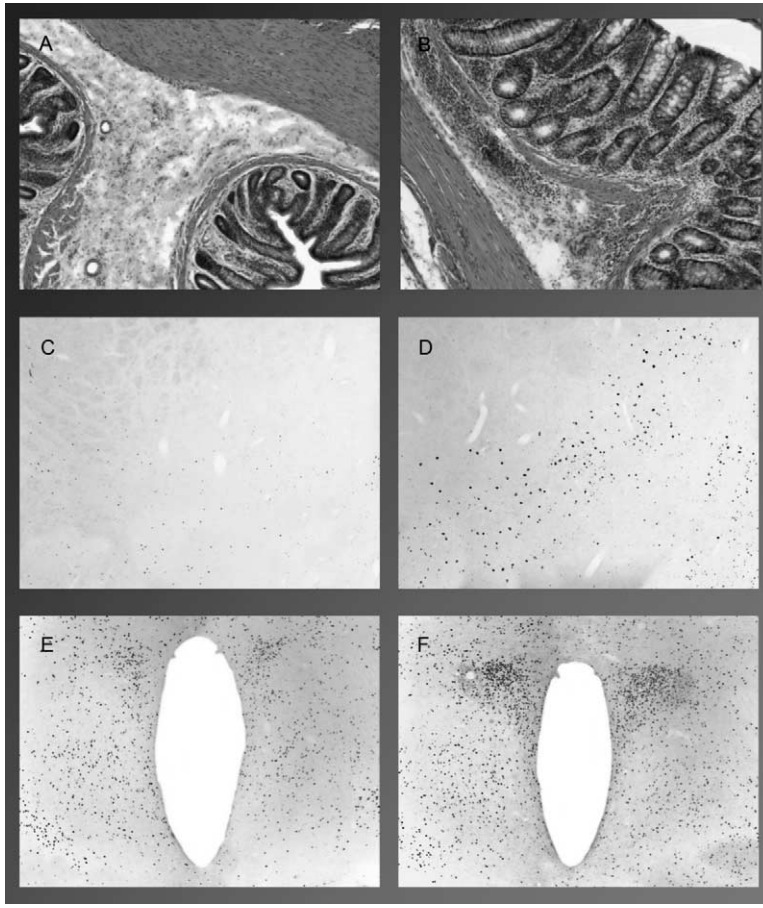

FIG. 5. Evidence showing brain and gut areas activated by visceral inflammation in a rat. Brain areas overlap with those often abnormal in autism, suggesting that inflammatory bowel disease (IBD) could be a model for testing treatments of autism. (A) Colon of control. (B) Colon showing infiltrates from TNBS-induced colitis. Concomitant cerebral metabolic activity patterns were compared by examining the regional distribution of c-*fos* gene expression. Colitis-induced changes localized to structures that are sensitive to stress and to secretin infusion by I.V. (C) Central amygdaloid nucleus of a control rat. (D) Colitis-induced stress reaction of central amygdala. (E) Hypothalamus of control rat. (F) Colitis-induced stress reaction of hypothalamus (Welch *et al.*, 2005).

were analyzed in healthy controls. Cortical and subcortical areas in control rats exhibited low constitutive c-*fos* expression (Fig. 5C and E). The IBD group exhibited robust c-*fos* activation of precognitive networks, specifically piriform, endopiriform/insular, central and medial amygdala (Fig. 5D), and paraventricu-

lar hypothalamic nucleus (Fig. 5F), midline intralaminar thalamus, and habenula. The brain activation patterns of IBD animals treated with secretin or oxytocin *et al.*, alone did not differ significantly from that of the untreated group of IBD animals.

We concluded from these results that visceral stress is processed centrally. In this study, experimental animals with IBD demonstrated activation of brain regions that control stress response patterns. Brain and gut areas affected in this study of acquired chronic visceral inflammation also overlap with those regions affected in autism. It is interesting to note that our IBD experiment provides evidence that contradicts one of Walter Cannon's five criticisms of the James' visceral theory of emotions (James, 1884) (Fig. 1A), namely that artificial induction of visceral changes typical of strong emotions does not produce corresponding emotions (Cannon, 1927) (Fig. 1B). As noted in our study, emotional brain regions were robustly activated by artificially induced visceral inflammation.

The overlap of brain areas concomitantly activated by visceral inflammation and those abnormal in autism suggests that IBD could be a model for testing treatments of autism. The affected areas express receptors for stress-regulatory peptides including secretin and oxytocin, which modulate the actions of classical transmitters (Gould and Zingg, 2003; Tay *et al.*, 2004). Maternal nurture behaviors such as breastfeeding and holding stimulate the release of brain/gut peptides, including secretin and oxytocin (Lucas *et al.*, 1980a, b; Matthiesen *et al.*, 2001). Deserving of study is whether combinations of peptides will be effective in resolving visceral inflammation and its neurological manifestations.

E. Visceral Inflammation Model of Autism

Although a direct pathophysiological link between autism and GI disorders has not been established, there is considerable evidence that visceral inflammation is co-morbid in autism (Ashwood *et al.*, 2004; Goldberg, 2004; Horvath *et al.*, 1999; White, 2003).

To date, it is not known whether the gut and/or the brain are primary sites of pathology in childhood developmental disorders. We presume, based upon clinical observations, that many chronic visceral and cerebral diseases share a common underlying peptidergic dysregulation, regardless of their sites of pathology. If this hypothesis is correct, one would expect peptidergic dysregulation to have an adverse impact on the body's ability to maintain homeostasis in the face of inflammation and to result in multiple brain/gut co-morbidities.

Chronic visceral disease generates pathological visceral activity patterns that transmit to emotional/visceral brain networks. The fact that neurological

manifestations occurred in brain regions abnormal in autism in both an induced (TNBS colitis) model and a genetic (IL-10-/- spontaneous colitis) model indicates that primary visceral pathology can potentially cause secondary pathological brain activity. (Welch *et al.*, 2003b, 2005).

Previous uses of animal models are relevant to the study of autism and related pathologies. In an animal model of hyperserotonemia, there were two important findings: oxytocin is low in the paraventricular hypothalamus and calcitonin gene related peptide (CGRP), a gut peptide belonging to the secretin family, is elevated in the amygdala (Whitaker-Azmitia, 2005). In addition to hyperserotonemia in autism, two other parallel findings are reported in humans: serum oxytocin is low in autism (Green *et al.*, 2001) and serum CGRP is elevated in neonates later diagnosed with autism (Nelson *et al.*, 2001).

In another animal model, autoimmune disease-sensitive SJL/J mice exposed to thimerosal, an ethyl mercury-containing preservative used in vaccines, showed behavioral changes and hippocampal neurodegenerative changes (Hornig *et al.*, 2004). Open to question is whether thimerosal vaccines are a risk factor for autism, given the high rates of autoimmune disease in autistic patients (Szatmari, 1999). Recent findings indicate that innate neuroimmune reactions play a central pathogenic role in inflammatory or neurodegenerative changes in some autistic patients (Vargas *et al.*, 2005).

A third animal model may pertain to autism and be useful in testing brain/gut pathology. In prenatally-stressed rats, brain-derived neurotrophic factor (BDNF) is reduced in the prefrontal cortex and striatum into adulthood, implying that adverse life events during gestation may interfere with the expression and function of this neurotrophin throughout development (Fumagalli *et al.*, 2004). BDNF, a molecular determinant of synaptic plasticity and cellular homeostasis, is important in central and visceral neurodevelopment and neuroprotection (El Shamy and Ernfors, 1997; Husson *et al.*, 2005). BDNF is abnormal in neonatal blood samples of infants later diagnosed with autism (Nelson *et al.*, 2001). Along with altered BDNF expression, post-mortem and neuroimaging studies demonstrate defective sensory as well as cerebellar/thalamic/cortical/striatal networks in autistic brains (Bauman and Kemper, 1985, 2005).

Sapolsky's vervet's (Uno *et al.*, 1989) are an excellent animal model of both gut and hippocampal pathology. Autistic children and vervets share non-compliance behaviors, social isolation, hippocampal deficits, and GI abnormalities, as cited in the previous section II. D. Long-term alterations of visceral sensitivity and gut mucosal integrity are found in animal models of maternal deprivation, one of the most profound forms of social isolation (Barreau *et al.*, 2004). Social isolation stress exacerbates both GI disorders and autistic spectrum disorders. Low nurture or social isolation predispose to gastric erosion and brain pathology in experimental models in areas abnormal in autism (Ackerman *et al.*, 1978; Andersen and

Teicher, 2004; Meaney *et al.*, 1988; Uno *et al.*, 1989). Socially insubordinate vervets and autistic children share behavioral, brain, and GI abnormalities (Breiner and Beck, 1984; Bauman *et al.*, 1985; Lightdale *et al.*, 2001; Uno *et al.*, 1989; White, 2003).

Using animals with gut inflammation to study central manifestations could be useful in learning more about the cellular response to homeostatic challenge. In particular, animal models of low/high-nurture offspring become more vulnerable to hippocampal neuronal loss via cell death, or apoptosis, when they receive low nurture (Weaver *et al.*, 2002). Offspring are protected against apoptosis when they receive high nurture, suggesting that high level nurture may protect against apoptosis. Forrester posited that when homeostasis is not restored, inflammatory responses persist and lead to apoptosis. Further, he states that this tissue response is universal, since all cells derive from the same blastocyst. Cell differentiation that results in different organ systems manifests itself in separate clinical disorders, obscuring the common underlying disease process (Forrester, 2004). This concept could explain the wide range of symptoms present in autism. Using a visceral inflammation animal model could help determine the extent to which cell arrest or cell death occurs in the brain as a result or the peripheral inflammation.

Such a model could also be useful in determining the extent to which neuropeptides will prevent or intervene in the process of cell arrest or apoptosis peripherally and/or centrally. We believe that apoptosis is the end stage of the cascade of adverse events that stems from unmodulated stress (Welch *et al.*, 2003a, 2004b, c, 2005). Whereas peripheral and central inflammation can stem from a wide variety of causes, in the case of developmental disorders we believe that peptidergic mechanisms may protect against cell arrest and apoptosis. Our clinical experience shows that it is possible to overcome the negative developmental effects of unmodulated stress via early intervention with intense maternal nurturing therapy. Development, including normal speech, cognition, and improved social interaction, proceeded rapidly following the therapy in some cases (Welch *et al.*, 2006). It is possible that some developmental cell groups had not suffered apoptosis, but rather were in a stage of cell arrest. The cells could have been activated by mechanisms of components of maternal nurture (Welch, 1987, 1988; Welch and Chaput, 1988; Welch *et al.*, 2005). A visceral inflammation animal model could be useful in testing whether this is in fact the case and whether change occurs simultaneously in the gut and the brain as we predict.

While the causal relationship between gut disturbance and autism remains in question, the use of an animal model of IBD presents a novel approach to understanding the multifactoral causes and comorbidities associated with complex developmental disorders.

IV. Future Directions

A. Neuropeptide Dosing in Future Clinical Trials

The amount, delivery methods and schedule of dosing are all factors that can have profound influence on the outcomes of clinical trials involving secretin and other peptides. We do not believe that a single peptide given as a single dose will be effective in re-establishing homeostasis. Rather, we believe that combinations of two or more peptides administered as a continuous infusion will more closely mimic the natural physiologic stress-adaptation pattern elicited by maternal nurturing and will ameliorate visceral inflammation and central activation.

The idea of single dosage in clinical trials of secretin seems to have followed from Horvath's single injection of secretin (Horvath *et al.*, 1998). Ferguson's finding that restoration of social recognition in oxytocin mutant mice can be achieved with one injection may also have encouraged a single dose protocol (Ferguson *et al.*, 2001). It is unreasonable to expect that effects from a single dose will have lasting effects. Indeed, Kuntz showed that the effects of secretin administration on glutamate and GABA diminish in just 2 hours (Kuntz *et al.*, 2004). It is more likely that long-range effects on stress adaptation response patterns will require continuous administration of peptides for days, weeks, or months. This fact should be kept in mind when assessing the results of studies that use single peptides or short-term administration of peptides. Whether multiple doses or continuous dosing would prolong the effects in humans needs further investigation.

B. Long-Term Goals of Our Research Efforts

We seek to demonstrate that combined secretin/oxytocin neuropeptide therapy is effective in ameliorating chronic developmental disorders of the brain/gut stress axis. Studies in progress focus on the brain, behavior, and circulatory/GI immune functions consequent to visceral inflammation before and after secretin/oxytocin treatment. The long-term effects of this combined peptide therapy may be demonstrated by reversal of predicted alteration of stress transmitters and peptides, as well as receptor binding sites abnormal in chronic mental and visceral metabolic disorders.

Clinical trials will assess whether combined secretin/oxytocin administration is effective in resolving visceral inflammation, autism, and autism with GI symptoms. Comparative neuroimaging studies will be designed to determine brain activation patterns of patients with IBD, patients with autism, and patients with co-morbid autism and symptoms of visceral inflammation.

Though some genetic and epigenetic risk factors for autism and inflammation have been established, we seek to determine a marker expressed by brain and gut that can identify a precursor stage of the diseases.

V. Conclusion

Our laboratory is engaged in efforts to translate bedside observations and experience into bench findings that will lead to a new paradigm in the treatment of developmental and behavioral disorders.

Our studies have demonstrated that secretin is synthesized in the hypothalamus, and that secretin activates visceral/emotional brain regions. We have also established that inflammatory bowel disease activates visceral/emotional brain regions in areas known to be abnormal in autism. Secretin and oxytocin may be secreted into or extracted from the blood/CSF, where interactions may occur with classical neurotransmitter systems, such as GABA, serotonin, and dopamine, and with other stress regulatory peptides such as CRH, vasopressin, and angiotensin.

The literature provides ample evidence that autistic children exhibit classic stress-induced inflammatory symptoms in brain and gut areas that are acted upon by secretin and oxytocin. Secretin and oxytocin are both important in determining stress response patterns and to maintaining homeostasis. The actions of secretin and oxytocin in the development and conditioning of stress adaptation networks via maternal nurturing are well documented. It follows that therapies intervening in the peptide mechanisms underlying maternal nurturing will be most effective in treating developmental disorders.

We believe developmental and behavioral disorders such as autism are the end result of an adverse cascade of events that stems from one or more genetic/environmental insults. Such insults can arrest the activation of key developmental programs. Over time, if uncompensated, the cascade can lead to maladaptive stress response patterns and to various interrelated psychological, neurological, and immunological pathology, including autism. Our clinical observations and the evidence presented in this chapter support the idea that it may be possible, regardless of etiology, to treat autism and other developmental and behavioral disorders effectively by intervening in stress mechanisms with exogenous combined secretin/oxytocin peptide treatment.

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IMMUNOLOGICAL FINDINGS IN AUTISM

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Autism is a disorder of neurobiological origin characterized by impairment of contact and communications. Typical symptoms of autism include extreme withdrawal and an abnormal absorption in fantasy, accompanied by delusion, hallucination, and an inability to communicate verbally or to otherwise relate to people. The cause of autism remains unknown. However, there are several factors including infectious, neurological, metabolic, environmental, and immunologic origin that have been thought to be involved in the disease development process of autism. The cellular entities playing a role in the pathologic processes in the autistic brain are the neurons, glial cells, endothelial cells, microglial cells, and astrocytes with blood brain barrier permeability playing an important role for the trafficking of the immune cells and mediators. In this chapter

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immunologic findings on autism are discussed. Particular emphasis is made on the aspects of immunological dysfunctions and inflammation as the two important immunological principles contributing to the diseases process in autism.

I. Introduction

The initial identification of autism dates back to 1943, when Dr. Kanner first observed a syndrome of abnormal neurological development and impaired social interactions, restricted stereotyped interests, and abnormalities in verbal and nonverbal behavior among several children. Kanner called this stereotypic behavioral disorder autism. Since then, the incidence rate in autism has increased and this disorder is currently one of the major pediatric health concerns in the United States. In 1997, the Centers for Disease Control and Prevention (1999) estimated that a broad definition of autism or autistic spectrum disorders (ASD) may be present in as many as one out of every 500 children. Studies in neuroimaging (Minshew *et al.*, 1993), anatomy, and cytotechnology (Bailey *et al.*, 1998a; Bauman and Kemper, 1994), and epidemiologic (Gillberg, 1990) findings suggest that ASD results from a variety of quantitative and qualitative abnormalities in brain structure. Some molecular, genetic, and cellular characteristics have been identified in cell types including the neurons, glial cells, endothelial cells, microglial cells, and astrocytes of the central nervous system.

Symptomatic manifestations of autism occur within the first 5 years of life and persist into adulthood. The neuropathological abnormalities in this disease have been largely confined to the cerebellum and medial temporal structures. Thus, their possible involvement in autistic development has been the subject of much interest. Several investigators reported cerebellar abnormality in autistic samples (Bauman and Kemper, 1994; Courchesne *et al.*, 1988, 1994; Ritvo *et al.*, 1986). However, some of these studies are debatable and need further confirmation (Bailey and Cox, 1996; Bailey *et al.*, 1998b). Furthermore, evidence for a decrease in cerebellar cell size with no differences in Purkinje cell densities between the normal and autistic children has been reported in literature (Fatemi *et al.*, 2002). Studies of Carper and Courchesne (2000), and Bailey and Cox (1998) have demonstrated that the degree of frontal lobe abnormality correlated with the degree of cerebellar abnormality. The frontal lobe appears to have an excess of neural tissue while the cerebellum has too little neuronal cells in autistic patients.

Even though the causes of autism remain debatable, some scientific findings provide further clues. Large/small brain size and volume, asymmetry in the right hemisphere, attention to details, overlooking the whole along with clumsy

behavior, and chronic inflammation in the central nervous system (CNS) are hallmarks of autism. Studies by Courchesne and his colleagues have shown that newborns who later develop autism have a smaller head size at birth but their head size grows rapidly between 1–2 months and 6–24 (Courchesne *et al.*, 2003). In addition, studies by Herbert and colleagues (2003) demonstrated that there is asymmetrical development of the brain's white matter in autistic children. The brain of children with autism seems to grow normally until age 9 months followed by a rapid period of white matter growth between the period 9–24 months (Courchesne *et al.*, 2003). Thus, in autism, there is asymmetrical-brain maldevelopment and potential abnormality/ies either how the brain is processing information or in the ability of the corpus callosum to network the two sides together where the right hemisphere is especially affected. In addition, studies from Just and his colleagues (2004) illustrated an alteration in brain circuitry causes the inability of autistic patients to utilize the right hemisphere of their brain that normally processes structures to recall the alphabet. However, autistic patients have good ability to appreciate details but little or no ability at conceiving the whole picture. This suggests an overconnectivity of local brain networks while long-range brain wiring are under-connected. Moreover, Teitelbaum and his colleagues (2004) showed that due to skewed brain wiring autistic subjects are clumsy and therefore use unusual strategies for locomotion. In conjunction with these reports is the finding of Goldberg (2000), who demonstrated that the parts of the cerebellum that govern the ability to restore balance operate normally in autistic children. Finally, Vargas *et al.* (2005) reported that the brain tissue of people with autism shows signs of chronic inflammation in the same areas that show excessive growth. The inflammation appears to last a lifetime with a characteristic increase in the number of astroglial cells. The brain areas that show hyperproliferation in white matter also show inflammation. There is also evidence for activated microglia in the spinal fluid (Vargas *et al.*, 2005). Thus, in autistic inflammation there is involvement of astroglial and microglial cells in the absence of lymphocyte infiltration or immunoglobulin deposition in the CNS. There is also increased production of pro-inflammatory and anti-inflammatory cytokines such as MCP-1 and TGFß-1 by neuroglia (Vargas *et al.*, 2005). All of these findings support a potential role for dysregulated immunoregulatory process and neuroinflammation in the CNS of patients with autism.

II. Immune Dysfunction in Autism

Substantial evidence suggests that the immune system plays an important role in the pathogenesis of autism (Bock *et al.*, 2002; Gupta, 2000; Wakefield *et al.*, 1998). While the exact mechanism of immune dysfunction in autistic patients

remains undefined, two general possibilities have been outlined. First, there might be a defect in immune regulation that causes hyper- or hypo-activation of the cellular components of the nervous system. This causes a homeostatic imbalance among the immunoregulatory factors in the brain and/or other affected organs such as the gastrointestinal tract. Second, an alternative mechanism of autistic development has been viewed as autoimmune reaction directed toward a specific target molecule in the brain.

A. Involvement of Neuronal Major Histocompatibility Complex (MHC) in Autism

Class I and Class II major histocompatibility complex were originally thought to be specific to immune cells, but are also expressed by various other cell types in the brain. In fact, certain allelic products of these genes have been thought to be associated with autism (Daniels *et al.*, 1995; Warren *et al.*, 1991, 1992), including the null allele of the C4B gene (located in the class III region of the MHC), the extended haplotype B44-S30- DR4 (the 44 allele of the HLA-B region, the S allele of the BF gene, the 3 allele of C4A, and the null allele of C4B and the DR4 allele) (Daniels *et al.*, 1995; Warren *et al.*, 1992, 1996). It has also been reported that the third hypervariable region (HVR-3) of certain DRb1 alleles has a very strong association with autism (Warren *et al.*, 1996). These observations provide evidence that MHC genes may be involved in autism.

Furthermore, accumulating evidence indicates that neuronal MHC Class I does not simply function in an immune capacity, but is also crucial for normal brain development, neuronal differentiation, synaptic plasticity, and even behavior. The observation that MHC exists not only in injured brain neurons (Neumann *et al.*, 1995, 1997; Wong *et al.*, 1984, 1985), but also in normal uninfected neurons (i.e., *in vivo*) opens up the possibility of it being involved in normal development as well as in diseases as in autism contributing to the pathophysiology of autistic development (Boulanger and Shatz, 2004). Class I molecules are expressed also by neurons that undergo activity-dependent, long-term structural and synaptic modifications including axonal branching and dendritic growth. In the adult hippocampus, MHC is required for normal long-term potentiation (LTP) and long-term depression (LTD), and is thought to be crucial to learning and memory building process (Boulanger, 2001). Thus, there is no room for immune molecules to reduce neural links (Helmuth, 2000).

Experimental studies with mutant mice genetically deficient in class I MHC or for a class I MHC receptor component, CD3 zeta showed an incomplete

refinement of connections between retina and central targets during development (Huh, 2000). In the hippocampus of adult mutants, N-methyl-D-aspartate receptor-dependent long-term potentiation (LTP) is enhanced, long-term depression (LTD) was absent, and specific class I MHC mRNAs were expressed by distinct mosaics of neurons. These results demonstrated an important role for Class I molecules in the activity-dependent remodeling and plasticity of connections in the developing and mature mammalian central nervous system (CNS). These results clearly show that MHC-I molecules are required for proper development in the CNS (Huh, 2000). Since the pattern of MHC expression is very diverse, it could be reasoned that the expression of MHC is directly related to the neuronal activity (Huh, 2000).

B. Impaired Cell-Mediated Immunity

Cell-mediated immunity is impaired in autism. This includes changes in the numbers and functions of macrophages, T cells, B cells, and natural killer cell activity (Gupta 2000; Warren *et al.*, 1986, 1987). In autistic patients who suffered from frequent gastrointestinal symptoms, Wakefield and colleagues demonstrated that CD3(+) cells were significantly increased in affected children compared with developmentally normal non-inflamed control groups ($p < 0.01$) reaching levels similar to inflamed controls (Ashwood *et al.*, 2003).

C. T-Cell Polarity in Autism

Helper T-lymphocytes have been shown to differentiate into two mutually regulatory subsets. Th1-like (IL-2, IFN-γ) mediates classical cell-mediated immune responses such as delayed-type hypersensitivity. Th2-like (IL-4, IL-6, and IL-10) cells promote humoral immune responses, in particular the production of IgE and IgG4 (human) or IgG1 (rodents). Over-activity of either cell type can result in a tissue-damaging autoimmune disease. A number of human diseases including asthma and some kidney diseases are thought to be caused by a Th-2 type autoimmune response. A shift occurs from T helper 1(Th1) to T helper 2 (Th2) T cells in autism as evidenced by a decrease in the production of inteleukin-2 (IL-2) and gamma interferon(IFN-γ), but there is an increase in the production of IL-4 (37). An imbalance of Th1/Th2 subsets of $CD4^+/CD8^+$ T cells towards Th2 may play a role in the pathogenesis of autism involving an autoimmune phenomenon (discussed later) (van Gent *et al.*, 1997).

D. Impaired Humoral Immunity

A number of studies have documented abnormal humoral responses in autistic individuals. Warren and collaborators (1997) found decreased serum IgA in 8 of 40 (20%) individuals with autism. Gupta (2000) has demonstrated that the immune system within autistics shows a tendency for upper respiratory tract infections, increased allergy and increased gut yeast infection, and the presence of parasites in some cases. He also found a link with serum immunoglobulin in autistics. These children had increased levels of IgM and IgE and low levels of IgA and IgG1 along with low antibody response to protein antigens (Gupta, 2000). It is also apparent that there is a low response from Th1 as its levels decrease and Th2 increases. It is assumed that if Th1 drops then the gut is open to viral infection and fungal overgrowth. Therefore, low IgA leads to poor gut protection. This results in lymphatic hyperplasia, due to altered self-antigens that may lead to auto-immunity. However, elevated levels of interleukin-12 and interferon-gamma (IFN-γ) are found in autism (Singh, 1996). It has been postulated by Singh that abnormal production of interleukin-12 (IL-12), a critical Th1 promoting cytokine, may be a compensatory mechanism in the body of autistic patients that leads to defective cell-mediated immunity and augmented humoral responses.

E. Brain-Specific Antibodies

Several antibodies reacting to brain tissue have been reported. Approximately 58% (19 of 33) sera of autistic children (less than or equal to 10 years of age) are found to be positive for anti-myelin basic protein (MBP) in autistic patients (Singh, 1993).

Autistic children, but not normal children, had antibodies to caudate nucleus (49% positive sera) implying that autoimmune reaction to caudate nucleus of the brain region may cause neurological impairments in autistic children (Singh and Rivas, 2004). The occurrence of autoreactivities to brain tissue in autistic patients may represent the immune system's neuroprotective response to a previous brain injury that may have occurred during neurodevelopment (Silva *et al.*, 2004). Antibodies against Purkinje cells and gliadin peptides were also observed in autistic patients (Vojdani *et al.*, 2004). There was further evidence that serum IgG anti-nuclear autoantibodies and IgM anti-brain endothelial cells antibodies were found in the sera of autistic patients (Connolly *et al.*, 1999). All these reports strengthen the hypothesis that there is an antibody response which cross-reacts with some component in the brain causing dysfunction of the affected area.

III. Role of Viral Infections in Autistic Development

Given the immunopathogenic features of autism, the development process of this disease is likely to include infection. In fact, it has been shown in neonatal rat infection with Borna disease virus, a neurotropic noncytolytic RNA virus, is associated with marked alterations in the cerebellum, along with reductions in granule and Purkinje cell numbers. In this infectious model, neurons are lost predominantly by apoptosis, by an increase in mRNA levels for pro-apoptotic products (Fas, caspase-1), a decrease in mRNA levels for the anti-apoptotic bcl-x and in situ labeling of fragmented DNA (Hornig *et al.*, 1999). The inflammatory infiltrates that accompany this infection are observed transiently in frontal cortex. Glial activation (microgliosis > astrocytosis) is prominent throughout the brain and persists for several weeks in concert with increased levels of proinflammatory cytokine mRNAs (interleukins 1alpha, 1beta, and 6 and tumor necrosis factor alpha) and progressive hippocampal and cerebellar damage (Hornig *et al.*, 1999).

Maternal exposure to a sublethal intranasal administration of human influenza virus (H1N1) in C57BL/6 mice in Day 9 corresponding to about the second trimester in humans has a very significant effect in the brain development. Prenatal exposure of pregnant mice with H1N1 virus has both short-term and long-lasting deleterious effects on developing brain structure in the progeny. This was evidenced by altered pyramidal and nonpyramidal cell density values, atrophy of pyramidal cells despite normal cell proliferation rate, and final enlargement of brain (Fatemi *et al.*, 2002).

A. Association of Measles Virus with Inflammatory Process in Autism

Maternal infection is a risk factor for many neurodevelopmental disorders, including autism (Ciaranello and Ciaranello, 1995; Patterson, 2002; Pletnikov *et al.*, 2002).

It was reported that 43% of mothers with an autistic child experienced upper respiratory tract, influenza-like, urinary, or vaginal infections during pregnancy compared to only 26% of control mothers (Comi *et al.*, 1999). Studies show that, in rats, maternal exposure to infection alters proinflammatory cytokine levels in the fetal environment, including the brain. It has been proposed that these changes may have a significant impact on the developing brain (Giralt *et al.*, 2002; Urakubo *et al.*, 2001). These observations suggest certain cases of autism may be a sequela of pathogenic infections, especially those of a viral origin (Ciaranello and Ciaranello, 1995; Hornig *et al.*, 2002; Pletnikov *et al.*, 2002).

The target sites for measles virus (MV) are similar to the sites affected by autism. These include the cerebellum, the hippocampus, amygdala, cingulate gyrus, hypothalamus, and the frontal and temporal lobes of the cerebral cortex. Although the route of infection by MV is respiratory, and despite its widespread dissemination to the skin, the intestinal tract and the nervous system are the organs affected. The virus has a strong predilection for lymphoid tissues in the early as well as late stages of the disease.

Human CD46 and CDw150 serve as two receptors for MV induced immunosuppression. CD46 molecule, a member of the complement regulatory cascade of proteins (Dorig *et al.*, 1993; Manchester *et al.*, 1994; Naniche *et al.*, 1993) is ubiquitously expressed on all nucleated cells (McQuaid and Cosby, 2002). CDw150 (signaling lymphocyte activation molecule, or SLAM) is a T-cell costimulatory molecule and is expressed only on immature thymocytes, activated and memory T cells, B cells, activated monocytes, and dendritic cells (Cocks *et al.*, 1995; McQuaid and Cosby, 2002; Minagawa *et al.*, 2001; Punnonen *et al.*, 1997; Sidorenko and Clark, 1993). These two receptors induce marked host immune suppression. Although monocytes express CD46, they are considerably resistant to MV. Once monocytes differentiate into immature myeloid dendritic cells (iDCs) (GM-CSF + IL-4-treated), the cells become susceptible to MV (Murabayashi *et al.*, 2002). DCs that matured via stimulation of their Toll-like receptors (TLRs) 2 and/or 4 exhibited an approximately fivefold increase in CDw150 at the protein level, resulting in higher levels of MV amplification in mixed culture of lymphocytes than in iDCs without TLR2/4 stimuli (Murabayashi *et al.*, 2002).

Measles stimulates maturation of antigen-presenting cells in skin, gut, and lungs. Measles also induces IL-6 from fibroblasts and interferon β and colony-stimulating activity from granulocytes and monocytes such as granulocyte-macrophage colony stimulating factor (GM-CSF) (Van Damme *et al.*, 1989). This consequently requires regulation of the immune system during future infections (Murabayashi *et al.*, 2002). Aberrant expression of TGFβ1 can stimulate inflammatory and fibrotic tissue formation and high intracellular TGFβ1 may induce over-expression of CD46 receptors, a portal for measles virus entry (Pasch *et al.*, 1999).

At the cellular level, MV causes cell cycle cessation, especially during the GO/G1 phase where major decisions regarding the cell's fate are determined (Schrag *et al.*, 1999). If the CD46 receptor is unavailable (Dorig *et al.*, 1993), then growth factor receptors (e.g., IGF-1 and epidermal growth factor (EGF) receptors) are used for viral entry (Schneider *et al.*, 2000). Immunologically, MV was found to be capable of suppressing immune responses (McChesney and Oldstone, 1989; Tsujimura *et al.*, 1998). Recent studies have suggested that MV infects and alters functions of T cells (Fugier-Vivier *et al.*, 1997; Hahm *et al.*, 2004; Niewiesk *et al.*, 2000) and antigen-presenting cells (APC) (Grosjean *et al.*, 1997;

Schnorr *et al.*, 1997; Servet-Delprat *et al.*, 2000). This infection skews the T-cell response to a Th2 phenotype (Griffin and Ward, 1993). MV generates type I interferon (IFN) that acts via a signal transducer and an activator of a transcription (STAT) 2-dependent, but STAT1-independent, pathway (Hahm *et al.*, 2005). Thus, it is possible that the MV contributes in autism by suppressing immune function.

IV. Role of Environmental Factors in Autistic Development

Occupational and/or environmental exposure to mercury is believed to harm human health possibly through modulation of immune homeostasis (Lawrence and McCabe, 2002). Several studies have demonstrated that imbalances in immune regulation by metals can lead to inadequate or excessive production of inflammatory cytokines (Gilmore, 2003; Croonenberghs *et al.*, 2002; Safieh-Garabedian *et al.*, 2004). Alternatively, metals can lead to inappropriate activation of lymphoid subsets involved in acquired immunity to specific antigens. Some resultant pathologies may include chronic inflammatory processes and autoimmune diseases. Metals may change the response repertoire by direct and indirect means by influencing expression of new antigens, new peptides, and/or may change antigen presentation by modifying the antigen-presenting complex (Lawrence and McCabe, 2002).

A. Mercury Link with Autism

Exposure to methyl mercury (MeHg) in high doses has profound effects on the CNS and can be fatal. Neuropathological studies indicate that the occipital cortex and cerebellum are most affected. Prenatal exposure studies from Japan and Iraq demonstrated diffused CNS damage with disruption of cellular migration (Bernard *et al.*, 2000; Choi, 1989). It has been hypothesized that postnatal exposure to thimerosal, a mercurial preservative added to the vaccines, may be associated with autism and learning/speech disorders. However, no direct test of this association has yet been reported (Tager-Flusberg *et al.*, 2000). No human studies as yet document any adverse effects of prenatal or early postnatal exposure to elemental mercury or mercury vapor (Davidson *et al.*, 2004).

A study by Vahter *et al.* (2000) examined the different species of mercury in the blood of pregnant women. They found high correlations between inorganic mercury levels in blood and urine during early pregnancy, a significant correlation between cord and maternal blood, and decreased mercury levels during

lactation—presumably the result of excretion in milk. The fetal brain is especially susceptible to damage from exposure to organic mercury.

Astrocytic swelling, excitatory amino acid (EAA) release and uptake inhibition, as well as EAA transporter expression inhibition, are known sequelae of MeHg exposure. The presence of Hg causes an inability of astrocytes to maintain control of the proper milieu of the extracellular fluid and, in turn leads to neuronal demise (Shanker *et al.*, 2003).

Heavy metals have been shown to exert immunotoxic effects on humoral immunity as well. IgG3 production is most sensitive to inhibition by mercuric chloride ($HgCl_2$) followed by IgG1 and IgG2b and then IgM and IgG2a. $HgCl_2$ exerts early, inhibitory effects on B-cell activation. This is manifested by the inhibition of RNA, DNA, and antibody synthesis. (Daum *et al.*, 1993). Metals by binding to SH radicals in proteins and other such groups, can cause autoimmunity by modifying proteins which via T cells activate B cells that target the altered proteins fibrillarin, a 34-kDa protein component of many small nucleolar ribonucleoprotein particles inducing autoimmunity. They also cause aberrant MHC II expression on altered target cells (Hu *et al.*, 1997a, b; Hultman *et al.*, 1994; Pollard *et al.*, 1997). Thimerosal (ethyl mercury) in individuals with pre-disposing HLA molecules bind to CD26 or CD69 and induce antibodies against these molecules (Vojdani *et al.*, 2003). Furthermore, the CD95/Fas apoptotic signaling pathway that is of critical importance in regulating peripheral tolerance, is disrupted by low and environmentally relevant concentrations of Hg^{2+} (McCabe *et al.*, 2005).

V. Inflammatory Mediators in Autism

Inflammation has an important repairing function, but, in CNS, frequently is the cause of damage. Usually neuroinflammation has the tendency to succumb to damage, which would explain the CNS pathology associated with autism (Chavarria and Alcocer-Varela, 2004).

The various components involved in the inflammatory response in the CNS include the participation of different cellular types of the immune system (macrophages, mast cells, T and B lymphocytes, dendritic cells), resident cells of the CNS (microglia, astrocytes, neurons), adhesion molecules, complement proteins, cytokines, and chemokines among other proteic components. Chemotaxis plays an important role in the recruitment of cells to the CNS. The lymphocyte recruitment implies the presence of chemokines and chemokine receptors, the expression of adhesion molecules, the interaction between lymphocytes and the bloodbrain barrier (BBB) endothelium, and their passage through the BBB to arrive at the site of inflammation (Little *et al.*, 2002). Recent studies by Vargas

et al., 2005) demonstrated that the two cells involved in inflammation are microglia and astroglia, which are essential for many neuronal functions. The presence of activated cells in their samples suggests that there is a chronic and a sustained neurological damage in autism. This activation could lead to abnormal function of neurons and synapses. Absence of any lymphocytes (T cells), plasma cells/antibody in the brain parenchyma suggests only the activation of astrocytes and microglial cells. These two cells are the classical players of innate immune response and thus demonstrated that an adaptive immunity is not playing an active role in autism. However, one cannot rule out the possibility of adaptive immunity playing a prominent role early in the prenatal or postnatal development phase of autism.

A. Proinflammatory Cytokines and Chemokines in Autism

As previously discussed, several lines of evidence indicate the immune system can influence the normal activities of the CNS. Cells of the immune system are present in the CNS where they show increased chemical activities under conditions of inflammation and disease. Cytokines and chemokines, secreted by either immune or non-immune cells, play critical roles in many chronic and acute inflammatory conditions. Therefore, it is likely that mediators of immune cell-CNS interactions under normal conditions and in diseased states are skewed. Indeed, studies from several laboratories provide evidence for an altered/unique cytokine profile in autism. Two pro-inflammatory chemokines, macrophage chemoattractant protein–1 (MCP-1), thymus, activation-regulated chemokine (TARC), and an anti-inflammatory and modulatory cytokine, TGF-ß1, were consistently elevated in the brain regions studied (Vargas *et al.*, 2005). MCP-1, a chemokine involved in monocyte and T-cell activation for trafficking into areas of tissue injury, was elevated both in brain parenchyma and CSF in cytokine protein array studies (Vargas *et al.*, 2005). In that study, immunochemistry revealed that astrocytes had infiltrated the cerebellum and cerebral cortex. The increased expression of MCP-1 in autism implies that it is linked to microglial activation and perhaps also to the recruitment of additional macrophages and microglia to areas of the cerebellum (Vargas *et al.*, 2005). There is evidence that MCP-1 may serve a signaling function in the damaged CNS that is distinct from its role in proinflammatory events (Little *et al.*, 2002). Its role in autism is not clear but its presence signifies inflammatory insult (Perrin *et al.*, 2005) or neuronal survival and protection (Uicker *et al.*, 2005). The observation that human fetal glial cells and their progenitors express specific receptors for chemokines and can be stimulated to produce MCP-1 as well as proliferate in response to chemokines, supports a role for these cytokines as regulatory factors during ontogeny (Rezaie and Male, 1999). MCP-1 expression in the cerebellum during prenatal

development suggests an association with maturation of Purkinje cells (Meng *et al.*, 1999). Like MHC-class II expression in microglia during CNS modeling, MCP-1 elevation in the brain of autistic patients may reflect persistent fetal patterns of brain development.

Other cytokines with pro-inflammatory and anti-inflammatory effect were also increased in the brain of patients with autism (Vargas *et al.*, 2005). An example of anti-inflammatory cytokines is TGF-ß1, a key anti-inflammatory cytokine involved in tissue remodeling following injury. Upregulating extracellular matrix proteins accomplish this. It can suppress specific immune responses by inhibiting T-cell proliferation and maturation while downregulating MHC class II expression especially in the brain stem (Johns *et al.*, 1992). In the study of Vargas *et al.* (2005), immunocytochemical studies, TGF-ß1 was localized mostly within reactive astrocytes and neurons in the cerebellum. Purkinje cells that exhibited microscopic features of degeneration showed marked reactivity for TGF-ß1. The elevation of this cytokine in autism may reflect a compensation mechanism to diminish neuroinflammation or remodel and repair injured tissue.

The prominent inflammatory cytokine profile was repeated in cerebrospinal fluid (CSF) as well in patients with autism (Vargas *et al.*, 2005). The marked increase of MCP-1 in CSF is indicative of pro-inflammatory pathway activation in the brain of autistic patients. This may be associated with activation of microglial cells as seen in brain parenchyma studies. These studies indicate that cytokine activation plays an important role in immune mediated processes and that their presence in the CSF in autistic patients may reflect an ongoing stage of inflammatory reactions. These reactions are associated with neuroglial activation and/or neuronal injury. The persistent elevation of cytokines in CSF also might reflect a neurodevelopmental arrest, as some of the cytokines are normally elevated during phases of neurodevelopment. Elevated levels of IL-6 are also observed in diseases associated with developmental disorders. IL-6 and cilliary neurotrophic factor (CNTF), a neuronal growth factor, share the same intracellular receptor. This suggests that IL-6 may influence the nervous system via pathways normally used by growth factors. Relatively little is known about the effect of IL-6 or chemokines on CNS neurons, the transduction mechanism linked to IL-6 or chemokine receptors, the pathways involved in IL-6, or chemokine induced neuropathology (Nelson *et al.*, 2004). In a small sample, Gupta and his colleagues (1998) found that tumor necrosis factor-alpha (TNF-α), another potent proinflammatory cytokine, was significantly increased in autistic populations. This finding was further corroborated in a study of Jyonouchi and collaborators (2001) who tested 71 autistic children aged 2–14 years and compared them with healthy siblings and other controls. In this study, innate immune responsiveness showed that in 59 of 71 (83.1%) autistic patients, lipopolysaccharide (lps) activated peripheral blood mononuclear cells (PBMCs) produced

levels of TNF-α, IL-1β, and/or IL-6 that were greater than 2 SD above the control mean (CM) values. Without stimulus, the basal level of proinflammatory/counter-regulatory cytokines was high in autistic patients. With stimulants phytohemagglutinin (PHA), tetanus, IL-12p70, and IL-18 of adaptive immunity PBMCs from 47.9 to 60% of autistic patients produced greater than 2 SD above the CM values of TNF-α depending on stimulants. The investigators concluded that a majority of the autistic children in their group exhibited excessive or poorly regulated innate immune responses especially involving increased TNF-α (Jyonouchi *et al.*, 2001). It should also be noted that although NO has been known to exert neuroprotective effects at low to moderate concentrations, NO becomes neurotoxic as the concentration increases. Excessive NO production can cause oxidative stress to neurons, ultimately impairing neuronal function and resulting in neuronal cell death (Abbott and Nahm, 2004). Indeed, plasma NO is high in some children with autism. This elevation may be related to IFN-γ activity (Sweeten *et al.*, 2004).

Taken together these studies suggest that several chemokines/cytokines and/or inflammatory mediators are involved in the pathogenesis of autism. However, their exact cellular source or mechanism of actions remains to be the subject of further investigations.

VI. Involvement of Toll-Like Receptors (TLRs) in Autism

The inflammatory signaling cascades leading to c-fos activation in glial cells have shown that activation by LPS in glial cells occurs via the serum response element (SRE) or cyclic AMP/calcium response element (CRE) in an independent manner, and involves the Elk1 or CREB/ATF-1 transcription factors. Elk1-mediated transactivation was dependent on p38 mitogen-activated protein kinase (MAPK), suggesting a crucial role of these factors in mediating inflammatory responses in the CNS (Simi *et al.*, 2005).

Additionally, Ozato *et al.* (2002) described the response of cell-surface toll-like receptors (TLRs) upon binding to microbial pathogens. There are at least 10 TLRs that recognize ligands from bacteria, viruses, yeast, and nucleic acids from viruses as well. There is a high binding specificity of the different TLRs for each microbial structure referred to as pathogen-associated molecular patterns (PAMPs) (Ozato *et al.*, 2002). The best studied is TLR4 that binds LPS from gram-negative bacteria. The ligation of LPS to cell surface TLR4 initiates a signal cascade that results in the activation of intracellular nuclear factor kappa beta (NFκB) and the transcription of numerous genes involved in immune responses. This signaling pathway appears to be common to all the TLRs whether the PAMPs originate from bacteria, virus, or yeast.

The central nervous system exhibits a similar immune reaction to pathogenic infection. There is a broad expression of TLRs in human brain astrocytes, oligodendrocytes, and microglia (Bsibsi *et al.*, 2002). Astrocytes and oligodendrocytes express mRNA for TLR2 that recognizes fungal, gram-positive, mycobacterial components and TLR3 that recognize, double-stranded RNA. Microglia cells express mRNA for a wide range of TLR family members (TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9) much like other cells of the monocytic lineage (Bsibsi *et al.*, 2002). The binding of LPS to TLR on microglia cells (brain macrophage) leads to the innate expression of cytokines, chemokines, extracellular matrix proteins, proteolytic enzymes, and complement proteins in the brain parenchyma (Aloisi, 2001; Nguyen *et al.*, 2002). It is also well established that glial cells participate in innate immune responses in human CNS (Nguyen *et al.*, 2002). The sharing of the TLR receptors between the astrocyte and microglia is another example where the neurology is communicating with immunology using common molecules. Microglial cells are the resident macrophage-like population in the CNS. Microglial cells remain quiescent until injury or infection activates the cells to perform effector inflammatory and antigen presenting cell (APC) functions. Mouse microglial cells express mRNA for all of the recently identified TLRs, TLR1–9. Furthermore, stimulation of quiescent microglia with various TLR agonists, including LPS (TLR4), peptidoglycan (TLR2), polyinosinic-polycytidylic acid (TLR3), and CpG DNA (TLR9) activated the cells to up-regulate unique patterns of innate and effector immune cytokines and chemokines at the mRNA and protein levels. In addition, TLR stimulation activated up-regulation of MHC class II and costimulatory molecules, enabling the microglia to efficiently present myelin Ags to CD4+ T cells. Thus, microglia appear to be a unique and important component of both the innate and adaptive immune response, providing the CNS with a means to rapidly and efficiently respond to a wide variety of pathogens (Olson and Miller, 2004).

VII. Autoimmunity in Autism

Inflammation has been linked with autoimmune insult. Aberrant innate immune response against endotoxin and immune reactivity to dietary proteins may be associated with apparent dietary product associated gastrointestinal inflammation in autistic children (Jyonouchi *et al.*, 2002, 2005). Another piece of information is the virus-induced autoimmune response to developing brain myelin that may impair anatomical development of neural pathways in autistic children (Singh *et al.*, 1993). The consequent anatomical changes of such autoimmune reactions could impair the nerve-impulse transmission and ultimately lead to life-long disturbances of higher mental functions (such as

learning, memory, communication, social interaction, etc.) that are seen in autistic populations.

A. Maternal Antibodies Can Trigger the Attack in Autism

Conceptually, it is possible that IgG from the mother can pass through the placental barrier and can react with antigenic proteins expressed on cell surface of lymphoid and/or neuronal tissues of the fetus and result in neuronal cell death. Since antigens expressed on lymphocytes are found on cells of the central nervous system and, perhaps, on other tissues of the developing embryo, it has been suggested that aberrant maternal immunity may be associated with the development of autism (Warren *et al.*, 1990). In fact, there is evidence in the literature supporting the importance of maternal antibodies in autism. Dalton and his colleagues (2003) have shown that serum antibodies that bind to rodent Purkinje cells and other neurons were detectable in a mother of three children: the first normal, the second with autism, and the third with a severe specific language disorder. The same serum when injected into pregnant mice during gestation produced altered exploration and motor coordination and changes in cerebellar magnetic resonance spectroscopy in the mouse offspring.

B. MMR Vaccination May Increase Risk Via an Autoimmune Mechanism

As previously mentioned, antibodies from autistic patients against MBP and neuron-axon filament protein (NAFP) cross-reacts with anti-measles antibody and human herpes-6 antibody (Singh *et al.*, 1998). This observation supports the hypothesis that a virus-induced autoimmune response may play a causal role in autism (Singh, 2000). Seventy-five of 125 (60%) autistic sera specifically detected measles hemagglutinin (HA) protein of measles-mumps-rubella (MMR) and over 90% of MMR antibody-positive autistic sera were also positive for MBP autoantibodies, suggesting a strong association between MMR and CNS autoimmunity in autism (Singh *et al.*, 2002). In another study, Singh and Jensen (2003) showed that there were elevated levels of measles antibodies in autistic children with no reaction to mumps or rubella.

There is some evidence that autism arises shortly after immunization with measles-mumps-rubella (MMR) and/or diphtheria-pertussis-tetanus (DPT) vaccines (Megson, 2000). Antibody levels to three vaccines, MMR, DPT, and DT (diphtheria-tetanus), were measured and it was found that the level of MMR antibodies was significantly higher in autistic children as compared to normal children (Singh *et al.*, 2002). There was a very high degree of specificity for MMR antibodies, particularly for measles (Singh *et al.*, 2002). The same result was also

found when monovalent measles vaccine was used instead of the trivalent MMR vaccine, furthermore pointing to a problem of only the measles subunit (Singh and Jensen, 2003). A high positive correlation (90% or greater) between the MMR antibody and the MBP autoantibody (Singh *et al.*, 2002) was detected. The deduction drawn from these studies is that the measles subunit of the MMR vaccine triggers an autoimmune reaction in a significant number of autistic children (Singh, 2000; Singh and Jensen, 2003; Singh *et al.*, 2002). MMR vaccine seems to induce interferon-gamma (IFNγ) only in breast-fed infants after primary measles immunization, a Th-1 cellular response. These results imply that the feeding pattern of infants can have a long-term effect on the immune modulation beyond weaning (Pabst *et al.*, 1991).

C. Potential Linkage of Environmental Factors with Autoimmune Events in Autism

Autoantibodies (primarily IgG) to neuronal cytoskeletal proteins, neurofilaments (NFs), MBP, were prevalent in male workers exposed to mercury. These findings were confirmed in rats and mice. There were significant correlations between IgG titers and subclinical deficits in sensorimotor function. Thus, peripheral autoantibodies to neuronal proteins are predictive of neurotoxicity, since histopathological findings were associated with disease damage. There was also evidence of astrogliosis (indicative of neuronal CNS damage) and the presence of IgG concentrated along the blood brain barrier (El-Fawal *et al.*, 1999). Autoimmune response to mercury has also been shown by the transient presence of antinuclear antibodies (ANA) and antinucleolar antibodies (ANolA) (Fagala and Wigg, 1992; Hu *et al.*, 1997; Nielsen and Hultman, 1999).

In an interesting study with newborns and thimerosol, autoimmune disease-sensitive mice were compared to strains resistant to autoimmunity. Mice were injected solely with thimerosal, a thimerosal-vaccine combination, or a saline solution. The comparative study showed growth delay, reduced locomotion, exaggerated response to novelty, and densely packed, hyperchromic hippocampal neurons with altered glutamate receptors and transporters in autoimmune mice. These animal studies implicate that impaired immunity might put some children at risk of developing autism after being exposed to thimerosal (Chian and Lipkin, 2004).

VIII. Summary

The immunopathogenesis of autism is presented schematically in Fig. 1. Two main immune dysfunctions in autism are immune regulation involving

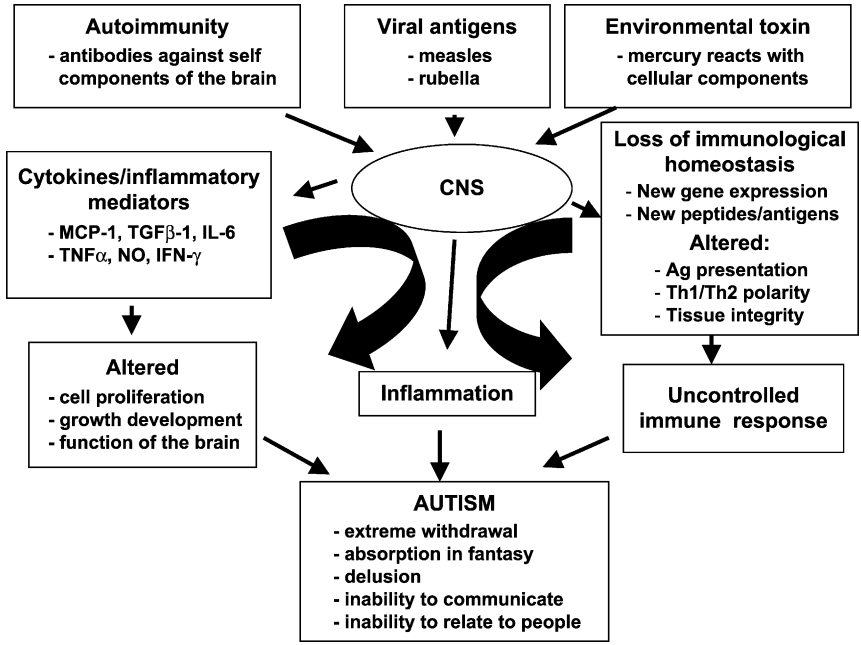

FIG. 1. Schematic presentation of immunopathogenesis of autism.

pro-inflammatory cytokines and autoimmunity. Mercury and an infectious agent like the measles virus are currently two main candidate environmental triggers for immune dysfunction in autism. Genetically immune dysfunction in autism involves the MHC region, as this is an immunologic gene cluster whose gene products are Class I, II, and III molecules. Class I and II molecules are associated with antigen presentation.

The antigen in virus infection initiated by the virus particle itself while the cytokine production and inflammatory mediators are due to the response to the putative antigen in question. The cell-mediated immunity is impaired as evidenced by low numbers of CD4 cells and a concomitant T-cell polarity with an imbalance of Th1/Th2 subsets toward Th2. Impaired humoral immunity on the other hand is evidenced by decreased IgA causing poor gut protection. Studies showing elevated brain specific antibodies in autism support an autoimmune mechanism. Viruses may initiate the process but the subsequent activation of cytokines is the damaging factor associated with autism. Virus specific antibodies associated with measles virus have been demonstrated in autistic subjects.

Environmental exposure to mercury is believed to harm human health possibly through modulation of immune homeostasis. A mercury link with the immune system has been postulated due to the involvement of postnatal exposure

to thimerosal, a preservative added in the MMR vaccines. The occupational hazard exposure to mercury causes edema in astrocytes and, at the molecular level, the CD95/Fas apoptotic signaling pathway is disrupted by Hg^{2+}. Inflammatory mediators in autism usually involve activation of astrocytes and microglial cells. Proinflammatory chemokines (MCP-1 and TARC), and an anti-inflammatory and modulatory cytokine, TGF-ß1, are consistently elevated in autistic brains. In measles virus infection, it has been postulated that there is immune suppression by inhibiting T-cell proliferation and maturation and downregulation MHC class II expression. Cytokine alteration of TNF-α is increased in autistic populations. Toll-like-receptors are also involved in autistic development. High NO levels are associated with autism. Maternal antibodies may trigger autism as a mechanism of autoimmunity. MMR vaccination may increase risk for autism via an autoimmune mechanism in autism. MMR antibodies are significantly higher in autistic children as compared to normal children, supporting a role of MMR in autism. Autoantibodies (IgG isotype) to neuron-axon filament protein (NAFP) and glial fibrillary acidic protein (GFAP) are significantly increased in autistic patients (Singh *et al.*, 1997). Increase in Th2 may explain the increased autoimmunity, such as the findings of antibodies to MBP and neuronal axonal filaments in the brain. There is further evidence that there are other participants in the autoimmune phenomenon. (Kozlovskaia *et al.*, 2000). The possibility of its involvement in autism cannot be ruled out. Further investigations at immunological, cellular, molecular, and genetic levels will allow researchers to continue to unravel the immunopathogenic mechanisms' associated with autistic processes in the developing brain. This may open up new avenues for prevention and/or cure of this devastating neurodevelopmental disorder.

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CORRELATES OF PSYCHOMOTOR SYMPTOMS IN AUTISM

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Stereotypical behaviors are defined as repetitive motor or vocal responses that serve no obvious adaptive function. The current diagnostic classification system, the DSM-IV-TR, includes the presence of stereotypical behaviors or interest in its criteria for autism. Research suggests that as many as 85% of children with autism exhibit repetitive behaviors or mannerisms. However, stereotypical behaviors are not specific to autism and are associated with other disorders such as Tourette's syndrome, schizophrenia, and mental retardation. Although the DSM-IV-TR criteria for stereotypical behaviors, as outlined in the diagnostic criteria for autistic disorder, focuses on motor symptoms that tend to occur in excess (e.g., twirling, spinning, head-banging), a broader conceptualization of the types of motor abnormalities observed in individuals with autism has been proposed more recently. Stereotyped patterns of behavior include not only excessive atypical movement but also the loss of typical movement (e.g., catatonia) in this broader definition. Support for this definition is evidenced by both clinical observations and empirical research. Research examining the overlap between catatonia and other stereotypic behaviors among individuals with autism suggest that the greatest risk for catatonic behaviors occurs in adolescence and may be precipitated by stressful events. Assessment tools for autism often include some measure of stereotyped behaviors and interest, but the presence of stereotypy is not in and of itself a pathognomonic sign of autism. Focusing primarily on the presence of classic stereotypical behaviors in diagnoses may subsequently lead to overidentifying autism in very young or mentally retarded individuals.

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A number of theories have been proposed over the years to explain the function and etiology of stereotypical behaviors. Lovaas and his colleagues, for example, proposed that the sensory and perceptual stimuli created through repetitive behaviors may be self stimulating. Others suggest that stereotypical behaviors are maintained by socially mediated positive and negative reinforcers; whereas biological theories focus on dysfunctions in the serotonin, opioid, and dopaminergic systems in the brain.

I. Introduction

Autism was first recognized as a pervasive developmental disorder (PDD) in the mid-twentieth century when Leo Kanner, M.D. noted 11 children engaging in atypical behaviors that were not consistent with other identified psychiatric conditions such as schizophrenia. Kanner observed three areas of impairment in these children including abnormal language, insistence on sameness, and social isolation, and is credited with labeling this constellation of symptoms as infantile autism. Wing and Gould (1979) noted similar symptoms approximately 30 years later and proposed the "triad of impairment," which conceptualized autism as a disorder characterized by impairment in reciprocal social interaction, verbal and non-verbal communication, and a restricted repertoire of activities or interests. The current diagnostic criteria for autism reflect these general categories and include qualitative impairment in social interaction (a minimum of 2 symptoms), communication (a minimum of 1 symptom), and a restricted repertoire and repetitive stereotyped pattern of behaviors, interests, or activities (a minimum of 1 symptom) with a total of 6 symptoms needed to meet full criteria [American Psychiatric Association (APA), 2000]. The current chapter focuses on symptoms characteristic of the latter category with an emphasis on the types of motor abnormalities manifested in children with autism, the function of these behaviors, and the underlying biological mechanisms to explain the occurrence of these behaviors.

Symptoms encompassing this latter category, including a restricted and stereotyped range of activities and interests, have typically been conceptualized as behaviors that are observable and atypical. The APA lists a cluster of symptoms representative of this category in the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition text revision (DSM-IV-TR; APA, 2000). The first symptom listed is a preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus. This may be manifested in seeking out and retaining vast amounts of information about one topic such as geography or presidents or collecting information about people's birthdates. It is important to note that up to 75% of children with autism also have comorbid

mental retardation and that this symptom tends to be more common in high functioning children with autism who do not score in the range of mental retardation on measures of intellectual functioning. A second symptom of this category is the compulsive adherence to nonfunctional routines and rituals. Children with autism may have a need to follow a particular route to school each day or complete daily tasks in a particular order. If they are prevented from engaging in their routine they exhibit distress which can escalate to physical resistance. A third symptom characteristic of this category as listed in the DSM-IV-TR is a preoccupation with parts of objects at the exclusion of recognizing and utilizing objects in a functional manner. A child with autism may be satisfied spinning the wheels of a toy car repetitively instead of rolling it on the ground or racing it with another car. Or a child may be preoccupied with and focus on the spinning blades of a ceiling fan or repetitively pressing buttons on a remote control rather than using the objects in a functional manner.

II. Stereotypic Behaviors

A fourth symptom characteristic of the restricted behavioral category is stereotyped or repetitive motor mannerisms. Stereotypic behavior has been defined as repetitive motor or vocal responses that serve no obvious adaptive function (LaGrow and Repp, 1984). Others define stereotypies as motor behaviors that are repetitive, topographically invariant, often rhythmical, and without purpose (Powell *et al.*, 1999). Regardless of the definitions used, they all share a common focus on the atypical nature and the repetitive quality of the behaviors. These behaviors have been recognized as a fundamental feature of Autism Spectrum Disorders (ASD) (APA, 2000; Lewis and Bodfish, 1998).

Empirical research has revealed that as many as 85% of children with autism exhibit repetitive behaviors or mannerisms (Volkmar *et al.*, 1986). Based on parental reports of a large sample of children with autism, the most common stereotypies observed are rocking (65%), toe-walking (57%), arm, hand, or finger flapping (52%), and whirling (50%). Repetitive behaviors can also include self-injurious behaviors such as head-banging, hand or finger-biting, hitting self with a closed or opened fist, hair-pulling, or scratching. These behaviors are often the focus of treatment for children diagnosed with autism because of the risk for physical harm.

In addition to reports of repetitive motor behaviors among children with developmental delays, these behaviors are commonly seen in normally developing children, occurring in up to 15–20% of pediatric patients (Matthews *et al.*, 2001). Stereotyped movements such as those observed in individuals with developmental delays have been reported to occur in as many as 3.5% of

typically developing young children (DeLissovoy, 1961). However, in typical children, these tend to be transient developmental phenomena, that usually disappear by age 5. Hence, stereotypical movements are not unique to autism. They are also observed in a number of different neurological and psychiatric disorders including Tourette's syndrome, Huntington's disease, Parkinson's disease, schizophrenia, and obsessive-compulsive disorder. Children with cognitive impairments, such as mental retardation (Lewis and Bodfish, 1998), and sensory impairments including visual and hearing impairments (Bachara and Phelan, 1980; Troster *et al.*, 1991) may also exhibit stereotypic behaviors. These symptoms, therefore, may simply be associated with severe cognitive impairment and, therefore, are not unique diagnostic features of autism (Volkmar and Lord, 1998). Matson and his colleagues (1996) refute this assertion with data revealing a significant difference in the number and intensity of stereotypic behaviors among adults with a dual diagnosis of autism and severe or profound mental retardation (75%) versus adults with severe or profound mental retardation *without* autism (7%). Similarly, Campbell and her colleagues (1990) found that all 224 autistic children included in their study exhibited some form of stereotypic behaviors. The most common types were lower body extremity movement (28%) and object stereotypy (25%). Between 15–20% also displayed upper extremity movement, hand flapping, or body rocking, and 12% engaged in head tilting behaviors. Children with more severe manifestations of autism tended to exhibit more severe stereotypical behaviors. These behaviors are often the focus of clinical attention because stereotypical movements can interfere with the acquisition of new behaviors and the application of previously learned skills (Epstein *et al.*, 1974; Koegel and Covert, 1972; Morrison and Rosales-Ruiz, 1997). They may also yield prognostic utility as they have been observed to lead to more severe self-injurious behaviors (Guess and Carr, 1991; Schroeder *et al.*, 1990).

A. Differential Diagnosis with Stereotypic Movement Disorder

Stereotypical movements are also characteristics of Stereotypic Movement Disorder (SMD), a disorder characterized by repetitive, nonfunctional motor behaviors that are severe enough to warrant treatment and interfere with daily activities or are of sufficient intensity to cause injury (APA, 2000). In the latter case, SMD can be coded as "with self-injurious behavior" per DSM-IV-TR. However, SMD is not diagnosed if the stereotypy is due to a compulsion, tic, PDD, or hairpulling. SMD is most commonly seen in individuals with mental retardation, and although it is seen in 2–3% of children and adolescents in community settings, it is much more common in adults with severe and profound mental retardation (MR) residing in institutional settings, where the prevalence is approximately 25% (APA, 2000; Rojahn *et al.*, 1998). SMD may develop

following a stressful or painful event and tends to peak in adolescence, followed by a gradual decline, although in severe/profound MR adults it may persist for years. In its most severe forms, SMD can be associated with self injury, including self biting, head banging, and other physical forms of self harm, which can be severe enough to cause physical damage (e.g., retinal detachment or blindness) if untreated. Self-injurious behavior (SIB) is sometimes observed among individuals with medical conditions including Lesch-Nyhan and Cornelia de Lange syndromes but can be present in the absence of a medical condition and warrant symptom-specific treatment because of the risk for physical injury.

B. Function of Stereotypic Behaviors in Autism

Hypotheses about the function of stereotypic behaviors in individuals with autism range from self-stimulation to communication. Lovaas *et al.* (1987) have suggested that the sensory and perceptual stimuli created through behaviors such as hand flapping or twirling are rewarding. Support for the stimulating benefits include the circumstances in which stereotypies occur, which is primarily when the individual is in an under-stimulating or over-stimulating environment. Hutt and Hutt (1968) observed that children with autism would increase their stereotypic behavior by more than 25% when their environment changed from an empty familiar room to a familiar room with an adult sitting quietly in the corner.

In a landmark study, Iwata *et al.* (1982) introduced functional analysis, an assessment methodology for testing hypotheses regarding contingencies for maintaining self injurious behavior. Since then, this methodology has been expanded and applied to numerous problem behaviors, including stereotypy (Durand and Carr, 1987; Repp *et al.*, 1988). Three main functional hypotheses for stereotypic behavior have emerged from this literature: sensory self stimulation, positive reinforcement, and negative reinforcement (Iwata *et al.*, 1994; Repp *et al.*, 1988). The sensory self-stimulation hypothesis is consistent with prior reports that stereotypy is maintained by access to reinforcing sensory and perceptual stimulation that may be a by-product of the stereotypic behavior itself. For example, repetitive eye poking may be maintained by access to visual stimulation, and repetitive behaviors such as rocking and spinning may be maintained by vestibular stimulation. This hypothesis suggests that stereotypy is *not* socially mediated. Rather, it appears that stereotypy is maintained by either positively or negatively reinforcing sensory consequences including access to pleasant visual, auditory, or vestibular stimuli or by the removal of aversive sensory stimulation, such as pain. Some researchers have subsequently suggested the term *automatic reinforcement* to explain maintenance of behaviors through non-social sensory mechanisms (Iwata *et al.*, 1994).

Behavioral functional analyses have also revealed that stereotypy is maintained by socially-mediated consequences (Durand and Carr, 1987; Kennedy *et al.*, 2000). In the case of positive reinforcement, this may occur in the form of delivery of social attention or access to preferred tangible items such as food or toys contingent upon the occurrence of stereotypic behaviors. The behaviors may be negatively reinforced by response-contingent escape from difficult task demands (Iwata *et al.*, 1994). Studies have found empirical support for the influence of social consequences in the maintenance of stereotypic behaviors among individuals with autism spectrum disorders (Durand and Carr, 1987; Mace and Belfiore, 1990) as well as successful application of treatments based on these functional causes (Repp *et al.*, 1988). Durand and Carr (1987) found that individuals with autism and other PDDs would engage in higher rates of rocking and hand flapping when presented with difficult tasks. These researchers showed that they could increase the stereotypics by making removal of the difficult task contingent upon the occurrence of these behaviors. When participants were taught to say "Help me" and provided with assistance in high demand situations, the rocking and hand flapping declined. Dawson and her colleagues (1998) have also observed that individuals with autism tend to engage in stereotypic, self-injurious, or aggressive behaviors primarily for sensory stimulation or to escape from social or work situations.

Others have suggested that stereotypic behaviors may serve as substitute behaviors in the absence of a preferred activity, or as a form of communication to gain access to or to remove particular types of environmental stimuli (Carr and Durand, 1985). Kennedy and his colleagues (2000) noted in their study of five children that stereotypical behaviors such as hand flapping, body rocking, and object manipulation occurred across a broad range of social settings including when demands were made of them and attention was given, when there were no demands or attention, and when the child was given a preferred toy or object. When taught an alternative and functional behavior such as a behavioral sign for "break" and "more," the children's stereotypic behaviors decreased and they exhibited more functional behaviors (Kennedy *et al.*, 2000).

C. Associated Motor Abnormalities and Catatonia in Autism

Although the DSM-IV-TR (APA, 2000) criteria for autism address motoric symptoms that occur in excess, a broader conceptualization that encompasses the absence of behavioral responses may better explain the motor abnormalities observed in autistic individuals. Leary and Hill (1996) proposed that the types of motor abnormalities classified under the third category of criterion A in the DSM-IV-TR (APA, 2000)—restricted repetitive and stereotyped patterns of behavior—might be more appropriately conceptualized as movement disturbances that "involve both the loss of typical movement and excessive atypical

movement" (p. 40). This definition would include difficulties with the dynamics of movement such as starting, executing, or switching movements. Such difficulties affect very simple movements such as head nodding to more complex movements such as coordinating verbal and non-verbal communication. Support for this broader definition includes the wide range of abnormal motor movements and paucity of movement in individuals diagnosed with autistic disorder. Clinical observations of children with autism have revealed catatonic behaviors including immobility, extreme negativism, mutism, and peculiarities of voluntary movements can often co-occur with symptoms of echolalia and bursts of hyperactivity (Ghaziuddin *et al.*, 2005). Dhossche (2004) describes three different case vignettes of individuals with PDDs who exhibit catatonic behaviors; one of which was an autistic adolescent male who was demonstrating aggressive outbursts and agitated behaviors along with such catatonic-type symptoms as psychomotor retardation, staring, waxy flexibility, and posturing. Ghaziuddin *et al.* (2005) describes a case vignette of an adolescent boy with autism who began exhibiting catatonic behaviors during adolescence. These behaviors included an excessive slowing of movements, progressive mutism, and a gradual loss of independent self-care activities. In the only large-scale empirical study to date examining catatonia in autism, Wing and Shah (2000) found that 17% of referrals (15 years of age or older) to a tertiary center for autism had severe exacerbation of catatonic features. These patients were more likely to have had impaired language and passivity in social interactions prior to the onset of the catatonic-like behaviors compared to patients without catatonic behaviors. Although there is limited research, the current literature suggests that individuals with autism are most at risk for developing catatonic symptoms during pre-adolescence and adolescence (Wing and Shah, 2000), possibly due to the stressors associated with adolescence that may affect the biological systems implicated in the development of stereotypic movements (e.g., dopamine).

Inspection of the diagnostic criteria for "catatonic features specifier" as set forth by the DSM-IV-TR (APA, 2000) reveals significant overlap in the criteria with psychomotor disturbances observed in some individuals with autism (e.g., posturing, stereotypies, alterations in the level of activity). Although this overlap is supported empirically (Bush *et al.*, 1996; Joseph, 1992; Rutter *et al.*, 1978, 1985; Wing, 1996), further investigation is warranted to understand the commonalities between these two disorders. Pending further research, it should not be assumed that all patients with autism who demonstrate behaviors consistent with catatonia warrant a comorbid diagnosis of catatonia. Rather, catatonic symptoms could be associated with other comorbid conditions such as depression or anxiety or due to medications as in the case of neuroleptic malignant syndrome (Takaoka and Takata, 2003). Nevertheless, patients who begin to show exacerbation of these symptoms or a significant decline in movement should be examined for catatonia (Ghaziuddin *et al.*, 2005).

D. Etiology of Stereotypies, and Associated Motor Disturbances in Autism

A number of biological theories have been proposed to explain the development of autism and more specifically the development of stereotypical behaviors in autism. Hypotheses include structural and neurochemical abnormalities as etiological explanations. Research examining these neuroanatomical explanations have typically relied on the findings from Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET). These findings suggest that there may be some abnormalities in the cerebellum and the neuronal systems that are directly influenced by the cerebellum including those that regulate attention, sensory modulation, autonomic activity, and behavior initiation (Courchesne *et al.*, 1988).

Of greater interest are biochemical findings for the etiology of autism and the implication of these findings for understanding the biological basis for stereotypical behaviors. As with many other psychiatric conditions, serotonin (5-HT) and the 5-HT system have been implicated as one of the biological processes that may contribute to the development of autism. Serotonin is involved in many aspects of human behavior including sleep, pain, motor function, appetite, and others (Volkmar and Anderson, 1989). Research examining 5-HT has consistently revealed that up to 50% of individuals with autism are hyperserotonemic (Geller *et al.*, 1982). The mechanism through which 5-HT influences the symptoms of autism, however, is still unknown. Findings specifically related to repetitive and stereotypic behaviors suggest that decreases in 5-HT levels are related to *decreased* stereotypies. Consistent with this hypothesis, Curzon (1990) found that rats administered acute treatment with agents that increase 5-HT activity were observed to engage in behaviors that resemble stereotypies (Curzon, 1990). However, contrary to this hypothesis, others have reported that when the reuptake of 5-HT is inhibited the rate of stereotyped behaviors tends to decrease (Powell *et al.*, 1997). Similarly, McDougle *et al.* (1996) observed an exacerbation of stereotypic behavior among a sample of adults with autism following a reduction of 5-HT through the depletion of its precursor, tryptophan. The effects of central versus peripheral 5-HT may differentially influence the manifestation of stereotypic behaviors.

Other neurochemicals, such as opioids, have also been implicated, specifically in the development of self-injurious and stereotyped behaviors. The opioid hypothesis suggests that when individuals engage in SIB, the brain releases neurochemicals such as endorphins that block pain and produce mild euphoria. Thus, individuals engage in self-injury and other forms of stereotypy to experience euphoria and escape/avoid painful stimulation. Although this may seem paradoxical, it is believed that continued self-injury actually blocks the painful stimulation that it would ordinarily produce, thus contributing to the maintenance of this behavior. Evidence to support this hypothesis includes both

animal and human studies. Research with rodents, for example, has revealed that self-injurious behaviors and stereotypies increase when opiate agonists are administered; whereas spontaneous stereotypies in farm animals were reduced or inhibited through opiate antagonists (Dantzer, 1986; Iwamoto and Way, 1977). Similar findings have been observed in human studies. Both Campbell *et al.* (1993) and Rojahn *et al.* (1998) reported a decrease in SIB following the administration of opiate antagonists (naltrexone). However, the declines observed by Campbell *et al.* were not statistically significantly.

More recent research has investigated the interaction between opiates, 5-HT, and the dopaminergic system to explain autism, and more specifically stereotypies and SIB. Serotonin receptors interact with the dopamine (DA) system and there is empirical evidence to suggest that stereotypical animal behaviors, such as head weaving, are decreased by lesions in the nigrostriatal and mesolimbic DA pathways and when DA blockers, such as haloperidol, are administered (Lewis and Bodfish, 1998). Additionally, there is evidence that the interaction between opioid and DA systems is an important mediator of abnormal or stereotyped behaviors (Lewis and Bodfish, 1998). For example, terminal fields that receive substantial amounts of DA innervation also contain large amounts of opioid peptides and receptors (Angulo and McEwan, 1994). The DA supersensitivity hypothesis proposes that repetitive and self-injurious behaviors result from low levels of DA in postsynaptic cells of the basal ganglia, resulting in supersensitivity of the post-synaptic receptors. Thus, the presence of small amounts of DA can be activating. Some support for this hypothesis can be seen in animal models of self-injurious and stereotypic behaviors in which these behaviors are induced following the administration of dopamine agonists such as L-Dopa (Lewis and Baumeister, 1982). Depriving animals of sensory stimulation and restricting their interactions with the environment in controlled laboratory experiments have been found to prevent DA innervation and subsequently produce spontaneous stereotypies (Martin *et al.*, 1991). Suomi and Harlow (1971) observed similar behaviors in non-human primates who had experienced early social deprivation. Interestingly, stereotypies were a long-term consequence of social deprivation in this research. It is hypothesized that early social deprivation or restricted environmental interaction results in a loss of dopaminergic innervation of important brain regions that results in DA receptor supersensitivity (Lewis and Bodfish, 1998).

Non-biological explanations for stereotypical behaviors include behavioral theories such as the reinforcement or communication-based theories. According to the reinforcement theory, there are four categories of reinforcement that maintain behavior. These categories are determined by whether the stimulus for the behavioral response is internal or external to the individual and whether a reinforcing stimulus is presented (positive) to precipitate the response or an aversive stimulus is removed (negative) to cease the response. Positive external

reinforcement is hypothesized to maintain a response such as a stereotypy with social attention, whereas positive internal reinforcement maintains repetitive behaviors through the production of pleasant internal sensory consequences (e.g., visual stimulation, vestibular stimulation, endorphin release). In contrast, negative external reinforcement is thought to maintain stereotypical behavior through the escape or avoidance of unpleasant conditions (e.g., task demands), whereas negative internal reinforcement maintains stereotypic or self-injurious behavior through the reduction or cessation of painful stimulation (e.g., otitis media) (Rojahn *et al.*, 1998).

A communication-based theory for stereotypical behavior asserts that these behaviors are functionally equivalent to attempts to communicate in nonverbal individuals. In the absence of language skills, these individuals are thought to have learned maladaptive behaviors as a means of communication. A child may engage in head banging, for example, to escape from task demands or to obtain social attention. That is, head banging may be functionally equivalent to saying "I want a break" or "I want attention" (Rojahn *et al.*, 1998). Studies have shown that efforts to train communicative alternatives (e.g., pointing to a sign that says "break") are effective in reducing self-injury in MR individuals (Carr and Durand, 1985).

To date, much of the literature on the etiology of movement disturbances in autism has focused on understanding the excesses of behavior (e.g., repetitive behaviors). With more recent attention given to a broader conceptualization of movement disorders in individuals with autism (Leary and Hill, 1996), researchers are beginning to investigate etiological explanations for the overlap between catatonia and autism. One neurochemical—gamma-aminobutyric acid (GABA)—has received some attention lately. Catatonia tends to respond well to treatment with benzodiazepines and other effective treatments for catatonia are known to enhance GABA functioning (e.g., barbiturates, ECT). Recently a GABA hypothesis has been proposed to explain autism (Dhossche *et al.*, 2002). While GABA is known to have an excitatory trophic role affecting neuronal wiring, organization, and plasticity of neuronal networks in early development, recent findings indicate that the effects of abnormal trophic GABA functioning in early development are consistent with the brain abnormalities reported so far in autistic individuals. At the present time, there is empirical evidence supporting overlapping etiologies for catatonia and autism including genetic studies implicating the long arm of chromosome 15 in both autism and catatonia (Borgatti *et al.*, 2001; Lauritsen *et al.*, 1999); however, further empirical investigations are needed to test hypotheses about the role of GABA in autism and catatonia before drawing conclusions.

E. Assessment of Stereotypy

Some of the common assessment measures for autism are the Autism Diagnostic Observation Schedule (ADOS; Lord *et al.*, 1989), the Autism Diagnostic Interview-Revised (ADI-R; Lord *et al.*, 1994), the Childhood Autism Rating Scale (CARS; Schopler *et al.*, 1988), and the Autism Behavior Checklist (ABC; Krug *et al.*, 1980, 1993). These measures also include items pertaining to stereotypic movements. The ADI-R is a structured interview that assesses functioning in three domains mirroring the diagnostic criteria for autism: Language and Communication, Reciprocal Social Interactions, and Restricted, Repetitive, and Stereotyped Behaviors and Interests. Items in the third domain include repetitive motor movements and self-injury. The CARS incorporates historical interview information from the parent and direct observation by a professional who rates the child's behavior in 15 domains. An item pertaining to "body use" measures the severity of stereotyped behaviors such as repetitive movements, rocking, spinning, and self-injury and an item pertaining to "object use" addresses inappropriate use of objects, which may include stereotyped use. The ABC is a 57-item parent or teacher rating scale that is included as part of the Autism Screening Instrument for Educational Planning-2. One of the five subscales, "Body and Object Use," contains several items measuring stereotypic motor movements, such as whirling, rocking, spinning, and flapping, as well as stereotyped use of objects (e.g., spinning or banging objects).

Research on differential diagnosis of autism has consistently found that very young children (under age 3) with mental retardation cannot be reliably distinguished from children with autism (Rutter and Schopler, 1987; Wing and Gould, 1979). This is because both groups exhibit language delays and social impairments, and have a great deal of overlap in restricted, repetitive, and stereotyped behaviors (Vig and Jedrysek, 1999; Wing and Gould, 1979). For instance, young children with mental retardation, adults with severe or profound mental retardation, as well as children with autism have all been shown to engage in hand stereotypies at varying rates (Vig and Jedrysek, 1999). Not surprisingly, some autism assessment measures, including the CARS and ABC, have been noted to have limitations in accurately diagnosing autism in children under the age of 3 (New York State Department of Health, 1999). The ADI-R and CARS have also overidentified very young (under 2 years) and mentally retarded children (mental age $<$ 18 months) (DiLavore *et al.*, 1995). Overidentification is further complicated by the fact that mental retardation can co-occur in 70–80% of children with autism.

III. Conclusion

Stereotypic behaviors and movement disorders are observed in individuals with autism and the DSM-IV recognizes this symptom as a diagnostic feature of autistic disorder. However, behavioral abnormalities of this nature are common early in development, and among children with mental retardation, other PDDs, and neurological conditions, thus limiting its specificity to autism. Diagnosing children with autism should, therefore, not be predicated on observations of stereotypic movements. Nevertheless, the occurrence of these behaviors and the interference they cause in the child's social and cognitive development has led to a body of research on the cause and treatment of stereotypic behaviors in individuals with autism. Etiological explanations for these abnormal movements are largely based on biological and neurochemical theories but may be reinforced, managed, and maintained by social and sensory consequences. More recently, movement disorders in autism have been expanded to include catatonia—a paucity of movement. Catatonic symptoms tend to appear in adolescence and may be precipitated by stressful events when observed in individuals diagnosed with autism. A different neurochemical process (e.g., GABA), may explain the lack of motor movements; however, further research is warranted before drawing definitive conclusions. Continued research will hopefully shed further light on the role of these movement disorders in autism.

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GABRB3 GENE DEFICIENT MICE: A POTENTIAL MODEL OF AUTISM SPECTRUM DISORDER

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Human chromosome 15q11–13 is associated with the neurodevelopmental disorders autism spectrum disorder, Angelman syndrome, and Prader-Willi syndrome. A number of genes have been identified within this region including a cluster of γ-amino butyric acid type A receptor subunit genes, GABRB3, GABRA5, and GABRG3 (encoding the β_3, α_5, and γ_3 subunits, respectively). Numerous studies have demonstrated the importance of the GABAergic system in neurodevelopment; therefore the presence of a group of $GABA_A$ receptor genes within this locus is intriguing. The β_3 subunit is widely expressed during the late embryonic to early postnatal period of brain development. A deficiency in the β_3 subunit during this critical period would be expected to negatively impact the temporal ordering of neurogenesis and synaptogenesis. This would have subsequent ramifications in the maturation of circuits involved in supporting complex behaviors, motor skills, and cognition. As expected, mice deficient in the

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gabrb3 gene exhibit a wide assortment of neurochemical, electrophysiological, and behavioral abnormalities, many overlapping with traits typically observed in autism spectrum disorder and Angelman syndrome. These findings suggest a potential involvement of the GABRB3 gene in the etiology of these neurodevelopmental disorders. The gabrb3 gene deficient mouse has proved to be a valuable model in the critical examination of the interconnection between development, pathology, and behavior as they relate to disorders of neurodevelopment.

I. Introduction

Autism spectrum disorder (ASD) results from a disruption in the rigid sequence of developmental events necessary for proper brain formation and function. Although the nature of this disruption has yet to be determined, there is a general consensus that idiopathic ASD is an oligomeric, multifactorial disorder (Pickles *et al.*, 1995) involving multiple genes (Risch *et al.*, 1999) with environmental factors likely contributing to the pathogenics of the wide continuum of observed phenotypic traits (Hornig and Lipkin, 2001; London, 2000). The high prevalence rate of ASD, 1 in 166 births (Fombonne, 2003), has prompted an urgency to develop animal models in advance of a comprehensive understanding of the molecular underpinnings associated with this disorder. However, establishing an acceptable animal model for any complex human disorder is a difficult task, as one cannot expect to fully replicate the human behavioral phenotype in a lower mammal. Therefore, the investigator must first ascertain that the animal model is valid for the intended disorder. A suitable animal model should possess (1) face validity, characteristics associated with the human disorder being investigated; (2) construct validity, similarities in the underlying etiology believed to be associated with the human disorder; and (3) predictive validity, use of the animal model to predict the potential outcome of a treatment regime applied to humans with the disorder. Consequently, Crawley (2004) has elegantly outlined a set of behavioral tasks, relevant to autistic-like behaviors, to serve as a guideline in which to establish the face validity of any proposed rodent model of ASD. The fundamental core symptoms of ASD center around three behavioral domains: (1) inappropriate social interactions; (2) poor communication skills; and (3) restrictive, repetitive (stereotypical) behaviors (Kanner, 1943; Rutter and Schopler, 1987). A broader, more variable ASD behavioral phenotype would likewise include anxiety (Gillott *et al.*, 2001), cognitive impairment (Vig and Jedrysek, 1999), epilepsy (Ballaban-Gil and Tuchman, 2000), motor deficits (Ghaziuddin and Butler, 1998), aggression (Cox and Schopler, 1993), sleep disturbances (Harvey and Kennedy, 2002; Limoges *et al.*, 2005), idiosyncratic

responses to sensory stimuli (Ayres and Tickle, 1980), attentional deficits (Allen and Courchesne, 2001), and hyperactivity (Gillberg *et al.*, 1996; Teitelbaum *et al.*, 1998).

Extensive evidence favors a role for GABAergic mechanisms in the etiology of ASD. As other chapters in this book address this issue, only a general overview will be given here in order to be succinct. $GABA_A$ receptors are the most ubiquitous neurotransmitter receptor in the mammalian nervous system. These heterooligomeric GABA-gated chloride channels are constructed from eight classes of subunits, some with multiple variants (α_{1-6}, β_{1-4}, γ_{1-3}, δ, ρ_{1-2}, π, ε, θ) (Olsen and DeLorey, 1999; Simon *et al.*, 2004). The various $GABA_A$ receptors isoforms display differing GABA sensitivities, distinct pharmacologies, and exhibit unique regional and temporal distribution patterns within the central nervous system. Interestingly, $GABA_A$ receptors are excitatory during development and primarily inhibitory in adulthood. During development, $GABA_A$ receptors, along with AMPA/Kainate glutamate receptors, mediate Ca^{2+}-dependent signal transduction pathways (Owens *et al.*, 1996) capable of influencing many brain developmental processes including proliferation, synaptogenesis, and circuit formation. Approximately 50% of all synapses in the developing cortex are GABA-releasing synapses, declining to 15% in the adult (De Felipe *et al.*, 1997).

A number of ASD susceptibility loci have been identified giving rise to the view that defects at several loci can result in overlapping phenotypes. Association studies suggest one of these ASD susceptibility loci is located within the chromosome 15q11–13 region (Bass *et al.*, 2000; Buxbaum *et al.*, 2002; Cook *et al.*, 1998; Martin *et al.*, 2000). Moreover, individuals with a maternal duplication of the 15q11–13 region are often diagnosed as having ASD (Martinsson *et al.*, 1996; Rineer *et al.*, 1998; Schroer *et al.*, 1998). Interestingly, maternal deficiency of the same 15q11–13 region results in the neurodevelopmental disorder Angelman syndrome (AS), a distinct syndrome, but with substantial phenotypic overlap with ASD (Steffenburg *et al.*, 1996). The core features of AS are severe mental retardation, epilepsy, ataxia, impaired language, and a happy demeanor (Williams *et al.*, 1995). The prevalence rate for AS has been reported to be as high as 1 in 10,000 live births (Petersen *et al.*, 1995). Interestingly, a deficiency of the paternal allele of 15q11–13 causes Prader-Willi syndrome (PWS), which exhibits a clinically distinct phenotype (mild mental retardation, obsessive-compulsive features, obesity, and hypogonadism) from that of AS (Cassidy and Morris, 2002). AS and PWS stem from a poorly understood phenomenon called genomic imprinting, involving a select group of genes expressed in the offspring from the chromosomal allele of only one parent instead of both, as is the usual situation for most genes. The above observations imply that the correct parental chromosomal gene(s) dosage from the 15q11–13 region is essential for normal brain development.

Progress in identifying ASD candidate gene(s) in the 15q11–13 region has been hampered by the high rate of recombination that occurs within this region, potentially masking linkage. However, several genes in the 15q11–13 region have been proposed as ASD candidate genes. These include the UBE3A (encoding a ubiquitin ligase), ATP10C (encoding a putative aminophospholipid translocase), and a cluster of $GABA_A$ receptor subunit genes, GABRB3, GABRA5, and GABRG3, (encoding the β_3, α_5, and γ_3 subunits respectively) (see Fig. 1). To date, evidence is lacking in support of the ATP10C gene's involvement in either ASD or AS. Whereas, a mutation in the UBE3A gene alone is capable of causing AS (Kishino *et al.*, 1997; Matsuura *et al.*, 1997). However, in most AS individuals the entire maternal 15q11–13 allele is deleted, resulting in a more robust epileptic phenotype than observed in AS individuals with just a mutation in the UBE3A gene (Minassian *et al.*, 1998). This has led to the suggestion that deficiencies in at least two genes, UBE3A and GABRB3, are likely required in order to manifest the more severe epileptic phenotype (Kishino *et al.*, 1997; Matsumoto *et al.*, 1992; Minassian *et al.*, 1998). In postmortem brain tissue taken from ASD cases, a reduction in both the UBE3A and GABRB3 gene products has been reported (Samaco *et al.*, 2005). However, association studies have reported mixed results for the UBE3A gene association in ASD (Nurmi *et al.*, 2001; Veenstra-VanderWeele *et al.*, 1999). Whereas, a strong association between the GABRB3 gene and specific subsets of ASD individuals has been reported (Nurmi *et al.*, 2003; Shao *et al.*, 2003). Further support for a GABAergic mechanism in the etiology of ASD comes from a case in which an individual with a rare autosomal-recessive disorder that prevented the proper synthesis of GABA, was likewise diagnosed with ASD, seizures and severe mental retardation (Burd *et al.*, 2000). Collectively, the previous observations and those presented in other chapters of this book provide a compelling argument that a developmental disruption in GABAergic mechanisms would adversely influence the sequence of events necessary to construct a properly functioning neural network.

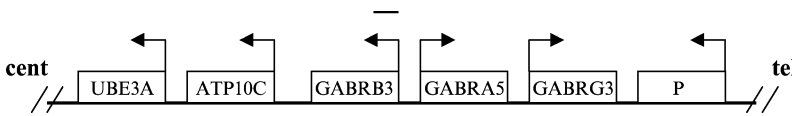

FIG. 1. Arrangement of several of the genes located within the human chromosomal region 15q11–13 (this gene arrangement is conserved on the mouse chromosome band 7B4). The chromosome 15q11–13 region is approximately 4 Mb in size. The maternal allele of this region is deleted in the majority of Angelman syndrome cases, whereas, the maternal duplication of this region often results in the individual being diagnosed with autism spectrum disorder. Association studies have also identified this region as being an autism spectrum disorder susceptibility locus. The black bar above the GABRB3 gene represents the 2.8 Kb region that was disrupted in producing the gabrb3 null mice (Homanics *et al.*, 1997). Cent: centromere, tel: telomere.

The following review examines a mouse line engineered to lack the expression of the gabrb3 gene (syntenic to the GABRB3 gene in human) (Homanics *et al.*, 1997). These mice exhibit decreased $GABA_A$ receptor density and high neonatal mortality rates, with survivors exhibiting a variety of abnormal neurochemical, electrophysiological, and behavioral features. Where appropriate, observations made in regards to these gabrb3 null mice will be correlated and contrasted with those observed in ASD and/or AS (see Table I). This mouse model has provided valuable insight into neurodevelopment and its disorders, in particular ASD and AS. In addition to the gabrb3 gene deficient mouse line, gene deficient mouse lines have been created for other genes found in the 15q11–13 region including the ube3a and gabra5 genes (Crestani *et al.*, 2002; Jiang *et al.*, 1998). In addition, GABA associated genes from other ASD susceptibility regions have likewise been disrupted. These include the Dlx1 and Dlx2 genes, important to the development of telencephalic GABAergic neurons (Anderson *et al.*, 1997; Marin and Rubenstein, 2001) and the reelin gene, encoding a protein important to the function of cortical GABAergic neurons (Alcantara *et al.*, 1998). Collectively, these mouse models will each contribute to a better overall understanding of the role of GABAergic mechanisms in ASD.

II. Molecular Characteristics of GABRB3 Null Mice

Gabrb3 deficient mice were produced by the targeted disruption of exons 1–3 of the gabrb3 gene by homologous recombination in 129/SvJ mouse embryonic stem cells and bred on a hybrid background (129/SvJ X C57Bl/6J) (Homanics *et al.*, 1997). This disruption was verified by northern blot indicating the complete absence of gabrb3 mRNA in homozygous null mice and a 30% reduction of gabrb3 mRNA in heterozygous mice. About 90% of the gabrb3 null mice die within 24 hrs of birth (Homanics *et al.*, 1997), similar to what has been observed in closely associated radiation-induced mouse mutants, p^{cp} mice, that lack the gabrb3 gene as well as the gabra5, gabrg3, and pink-eyed dilution (p) genes, however, sparing the ube3a gene (Lyon *et al.*, 1992; Nakatsu *et al.*, 1993).

III. Morphology

The high mortality rates exhibited by homozygous gabrb3 null mice and p^{cp} mutant mice are likely due to the high incidence of cleft palate, common in these mouse mutants. Cleft palate occurs in about 57% of the gabrb3 null mice (Homanics *et al.*, 1997) and 95% of the p^{cp} mutant mice (Nakatsu *et al.*, 1993).

TABLE I

QUALITATIVE COMPARISONS BETWEEN AUTISM, ANGELMAN SYNDROME, AND gabrb3 NULL MICE

Characteristic	Autism	Angelman syndrome	gabrb3 null mice	Reference
Impaired social interactions	+	+	poor maternal care	Gillberg *et al.*, 1996; Penner *et al.*, 1993; Homanics *et al.*, 1997
Repetitive, stereotypical behavior	+	+	+	Barthelemy *et al.*, 1997; Summers *et al.*, 1995; DeLorey *et al.*, 1998
Poor motor coordination	+	+	+	Barthelemy *et al.*, 1997; Williams *et al.*, 1995; Homanics *et al.*, 1997
Hyperactivity	+	+	+	Barthelemy *et al.*, 1997; Summers *et al.*, 1995; DeLorey *et al.*, 1998
Tactile hyperresponsivity	+	hyperreflexive	+	Ayres and Tickle, 1980; Viani *et al.*, 1995; Ugarte *et al.*, 2000
Sensitivity to temperature	+	?	+	Harrison and Hare, 2004; Ugarte *et al.*, 2000
Cognitive impairment	+	+	+	Vig and Jedrysek, 1999; Williams *et al.*, 1995; DeLorey *et al.*, 1998
Craniofacial dysmorphism	+	+	+	Wolpert *et al.*, 2000; Williams *et al.*, 1995; Homanics *et al.*, 1997
Reduced benzodiazepine binding				
–Hippocampus	+	+	+	Blatt *et al.*, 2001; Holopainen *et al.*, 2001; Sinkkonen *et al.*, 2003
–Cerebellum	+	+	newborn	Pfund *et al.*, 2001; Holopainen *et al.*, 2001; Homanics *et al.*, 1997
Sleep disturbances	+	+	+	Barthelemy *et al.*, 1997; Williams *et al.*, 1995; Wisor *et al.*, 2002
Epilepsy				
–Age dependent evolution	+	+	+	Rossi *et al.*, 1995; Matsumoto *et al.*, 1992; DeLorey *et al.*, 1998
–Myoclonic jerks	+	+	+	Matsumoto *et al.*, 1992; Guerrini *et al.*, 1996; DeLorey *et al.*, 1998
–Multiple seizure types	+	+	+	Carod *et al.*, 1995; Minassian *et al.*, 1998; DeLorey *et al.*, 1998
–Worsens with carbamazepine	one case	+	+	Monji *et al.*, 2004; Guerrini *et al.*, 2003; DeLorey *et al.*, 1998
–Ethosuximide is beneficial	?	+	+	Sugiura *et al.*, 2001; DeLorey *et al.*, 1998
–Slow wave EEG abnormalities	+	+	+	Hughes and Melyn, 2005; Minassian *et al.*, 1998; DeLorey *et al.*, 1998
–Paroxysmal EEG abnormalities	+	+	+	Rossi *et al.*, 1995; Kumada *et al.*, 2005; DeLorey *et al.*, 1998

+ effect reported, ? not tested.

The higher penetrance of cleft palate in p^{cp} mice, compared to gabrb3 null mice, is likely due to either a difference in genetic backgrounds of the two strains or to the additional loss of genes in the p^{cp} mouse. A significant proportion of the 43% of gabrb3 null mice born without an obvious cleft palate likewise die within the first day and are described as having neonatal feeding difficulties. Survivors are runted until weaning, achieving normal body size by adulthood, and are fertile but have somewhat reduced lifespans. Cleft palates also arise in mice deficient in the gad67 gene (encoding gamma-amino decarboxylase that synthesizes GABA) as well as mice prenatally exposed to drugs that alter GABA signaling (Condie *et al.*, 1997; Jurand and Martin, 1994). Additional studies have likewise implicated GABAergic mechanisms in normal palate development (Culiat *et al.*, 1995). The gross morphology of the brain is normal in gabrb3 null mice (Homanics *et al.*, 1997) and p^{cp} mutant mice (Nakatsu *et al.*, 1993). However, detailed assessments of brain morphology has not been performed in these mouse mutants.

Although cleft palates are rare in ASD and AS, craniofacial dysmorphic features such as protruding jaws, wide-spaced teeth, large mouths, and feeding difficulties in infancy are characteristic of AS (Williams *et al.*, 1995) whereas, high-arched palates are reported in 40% of ASD cases associated with the inverted duplication of chromosome 15q11–13 (Mann *et al.*, 2004; Wolpert *et al.*, 2000).

IV. Neurochemistry

Whole brain homogenates from newborn gabrb3 null mice exhibited a 50% reduction in both [^{3}H]muscimol binding to the GABA binding site and [^{3}H]Ro15-4513 to the benzodiazepine binding site on the $GABA_A$ receptor, as compared to whole brain homogenates taken from wildtype littermates (Homanics *et al.*, 1997). The same study also reported a 50% reduction in [^{3}H]Ro15-4513 binding in whole brain homogenates taken from adult gabrb3 knockout mice compared to adult wildtype mice. Brain slice autoradiography from 1 day old newborn mice provided a more insightful assessment of [^{3}H]Ro15-4513 binding in various brain regions including a significant reduction in binding to hippocampus (40%), thalamus (49%), frontoparietal cortex (30%), caudate/putamen (25%), and olfactory bulb (59%) (Homanics *et al.*, 1997). In a separate study in which the above brain regions were assessed in adult gabrb3 knockout mice, and compared to wildtype littermates, a significant reduction in [^{3}H]Ro15-4513 binding was observed in hippocampus (45%), cerebral cortex (32%), and caudate/putamen (32%) with non-significant reductions in thalamus (28%) and the granule and molecular layers of the cerebellum (11% and 5%, respectively) (Sinkkonen *et al.*, 2003). The consequence of this reduction in $GABA_A$ receptor binding density in regards to

electrophysiological function and behavior will be addressed in the following sections.

Neurochemical assessments of ASD and AS brains, likewise, provide support for a role of $GABA_A$ receptors in these disorders. For example, single photon emission computer tomography revealed a 60% reduction in the binding of the benzodiazepine radioligand [^{123}I]iomazenil in the cerebellum of an AS patient, with a deletion of the maternal allele of chromosome 15q11–13, compared to three normal control subjects (Odano *et al.*, 1996). Additionally, a Positron Emission Tomography (PET) study also detected significant bilateral reductions in binding of the benzodiazepine radioligand [^{11}C]flumazenil in cerebellum (34–43% reduction) and hippocampus (18–22% reduction) of three separate deletion AS cases as compared to a non-deletion AS case (UBE3A gene mutation) (Holopainen *et al.*, 2001). In a PET study focusing on ASD, four out of the nine ASD children examined were found to exhibit a significant reduction in whole brain [^{11}C]flumazenil binding with focal decreases in the cerebellum as compared to eight age-matched children with temporal lobe epilepsy and seven normal adults (Pfund *et al.*, 2001). Significant reductions in binding density of the benzodiazepine radioligand [^{3}H]flunitrazepam (15–35%) has also been observed in hippocampus from four ASD cases compared to three controls (Blatt *et al.*, 2001). Another six receptor densities (5HT1A, 5HT2, M1, NMDA, kainate, choline uptake sites) were also assessed in these brains without significant differences being observed. Lastly, a significant reduction in the expression of the β_3 subunit protein was reported in postmortem cerebral cortex in five out of nine (56%) ASD cases, two out of three (66%) Rett syndrome cases (an ASD subtype), and two out of two (100%) AS cases relative to 11 control brains (Samaco *et al.*, 2005). Taken together the previously mentioned studies provide compelling evidence for a reduction in the density of $GABA_A$ receptors in both ASD and AS brains.

V. Electrophysiology

A. Dorsal Root Ganglia

Electrophysiological recordings from sensory neurons isolated from the dorsal root ganglia (DRG) of neonatal gabrb3 null mice revealed a dramatic decrease (~80%) in the maximal amplitude of GABA-activated chloride currents in a population of these neurons compared to those taken from wildtype siblings (Homanics *et al.*, 1997). The same study also found sensory neurons taken from heterozygous mice lacking one allele of the gabrb3 gene, likewise exhibited a statistically significant reduction (25%) in GABA-activated current amplitude,

relative to neurons taken from wildtype mice. These findings are in agreement with mRNA expression studies on DRG cells that suggest that mRNA for β_3 subunit is the predominant β subunit expressed (Ma *et al.*, 1993). Pharmacological studies in sensory neurons taken from both gabrb3 null mice and control littermates further indicate there is little or no compensation for the loss of the β_3 subunit by the other two β subunits (Krasowski *et al.*, 1998). $GABA_A$ receptors on the terminals of sensory afferents in the dorsal and ventral horn of the spinal cord are expected to provide presynaptic inhibition (Eccles *et al.*, 1961). Therefore, it has been suggested that some of the motor manifestations of the hyperexcitability/hyperresponsivity seen in the gabrb3 null mice may be due to the ineffectiveness of spinal presynaptic inhibition resulting from the decline in $GABA_A$ receptor expression on the terminals of primary afferents (Homanics *et al.*, 1997). As direct assessment of DRG electrophysiology in individuals with ASD or AS is not possible, this mouse model provides a means by which to study the consequences of impaired GABAergic inhibition in the DRG. If GABAergic inhibition were compromised in the DRG of ASD and/or AS individuals one would expect these individuals to be hyperresponsive to sensory input. Consequently, there is evidence that ASD individuals are hyperresponsive to touch (Ayres and Tickle, 1980).

B. Thalamus

Electroencephalographic oscillations that occur during sleep (partial synchronous oscillations) and those that occur during absence epilepsy (hypersynchronous oscillations) result from synchronous activity in the cerebral cortex through interactions with inhibitory circuits arising in the reticular thalamic nucleus (RTN) and the thalamocortical relay nuclei (Huguenard and Prince, 1994; Steriade *et al.*, 1993; Warren *et al.*, 1994). Inhibitory postsynaptic currents (IPSCs) in RTN neurons are mediated by $GABA_A$ receptors that likely contain the β_3 subunit, as this is one of the limited $GABA_A$ receptor subunits found in this nucleus (Pirker *et al.*, 2000; Wisden *et al.*, 1992). By examining the inhibitory function of the RTN in thalamic slices taken from gabrb3 null mice, one can assess whether intra-RTN inhibition is suppressed, thereby promoting intrathalamic synchrony. GABA mediated inhibition and spontaneous IPSCs were observed to be nearly abolished in the RTN of gabrb3 null mice, resulting in a dramatic intensification of the oscillatory synchrony (Huntsman *et al.*, 1999). The same study found GABA mediated inhibition and spontaneous IPSCs to be unaffected in the thalamic relay neurons of the ventrobasal complex, which contains other β subunits. The previous findings demonstrate how the inactivation of inhibitory postsynaptic $GABA_A$ receptors in the RTN can alter oscillatory functions with the likely consequence of disrupting sleep architecture

(McCormick and Bal, 1997) and causing epilepsy (Huguenard and Prince, 1994); both features are observed in gabrb3 null mice (DeLorey *et al.*, 1998; Homanics *et al.*, 1997; Wisor *et al.*, 2002) as well as in ASD (Limoges *et al.*, 2005; Rossi *et al.*, 1995) and AS (Bruni *et al.*, 2004; Minassian *et al.*, 1998). It is noteworthy that the RTN is also involved in attentional processing (Guillery *et al.*, 1998), a feature often reported as being impaired in ASD (Allen and Courchesne, 2001).

C. Hippocampus

In situ hybridization studies demonstrate there to be a substantial amount of mRNA from the gabrb3 gene present in CA1 neurons of the hippocampus (Laurie *et al.*, 1992). Hippocampal neurons isolated from neonatal gabrb3 null mice and cultured for 4–5 days exhibited significant reductions (50%) in the maximal amplitude of GABA-evoked whole cell currents when compared to hippocampal neurons from wildtype littermates (Krasowski *et al.*, 1998). This is in agreement with the 40% reduction in [^{3}H]Ro15-4513 binding to hippocampus from neonatal gabrb3 null mice compared to wildtype controls, discussed earlier (Homanics *et al.*, 1997). $GABA_A$ receptor function is less impaired in the hippocampal neurons of the neonatal gabrb3 null mice, compared to RTN neurons. This is likely reflective of the larger proportion of $GABA_A$ receptors in the hippocampus that contain either the β_1 or β_2 subunits than is found in the RTN (Pirker *et al.*, 2000), making the hippocampus less dependent on the β_3 subunit. A reduction of GABAergic inhibition in the hippocampus would be expected to cause cognitive deficits and contribute to a heightened seizure susceptibility, both of which are observed in the gabrb3 null mice (DeLorey *et al.*, 1998; Homanics *et al.*, 1997) as well as in ASD (Rossi *et al.*, 1995; Vig and Jedrysek, 1999) and AS (Williams *et al.*, 1995).

D. Olfactory Bulb

The gabrb3 gene is abundantly expressed in the rodent olfactory bulb (OB) with the granule cells expressing only the β_3 variant of the β subunit and the principal cells (mitral and tufted cells) expressing all three β subunits (Laurie *et al.*, 1992; Nusser *et al.*, 1999). In the olfactory bulb, odors trigger synchronous oscillatory activity that is believed to arise from coherent and rhythmic discharges of large numbers of neurons (Lagier *et al.*, 2004). The gabrb3 null mice exhibited a >93% reduction in GABA-mediated synaptic inhibition of granule cells, the local inhibitory interneurons of the OB, as well as an augmentation of inhibitory postsynaptic currents in the principal cells. These two effects lead to an increase in network oscillations in the OB resulting in complex effects on olfactory

learning, representation, and discrimination (Nusser *et al.*, 2001). Consequently, the same study found the gabrb3 null mice to be better at discriminating a particular odor from closely related odors but poorer at discriminating closely related mixtures of odors than their wildtype littermates. Sensory symptoms associated with ASD, including olfaction, have been less studied than those dealing with social and cognitive functioning. However, a study employing a parental questionnaire of sensory reactivity reported that ASD children (2–4 yrs old) exhibited significantly higher scores on taste/smell sensitivity than normal children of the same age (Rogers *et al.*, 2003). It is not clear how one would best go about correlating the previous findings in mice with those found in ASD, as rodents rely more on their sense of olfaction than do humans. A more in depth study of olfaction in ASD individuals would be required before one could assess whether gabrb3 null mice model any of the olfactory abnormalities observed in ASD. To date no study of olfaction in AS has been performed.

VI. Epilepsy

A. Seizures and EEG Abnormalities

Virtually all gabrb3 null mice experience some form of recurring spontaneous seizure starting at about 10 weeks of age and becoming more frequent as they age. Seizure severity ranged from twitching of mouth muscles, face, whiskers, and ears to the more robust seizures that involved head and bilateral forelimb jerks, arching of the back, straub tail, and the mouse falling on its side. The severest observed seizures involved strong clonic shaking that progressed into a wild running/bouncing phase (DeLorey *et al.*, 1998). Seizures were usually followed by a period of behavioral quiescence. Seizures were also noted in mice heterozygous for the gabrb3 gene, although with less frequency than observed in the homozygous gabrb3 null mice (DeLorey *et al.*, 1998; Homanics *et al.*, 1997). EEG measurements performed on gabrb3 null mice revealed an evolving electrocortical phenomena in which young (<10 weeks old) gabrb3 null mice display relatively normal EEG traces that became markedly abnormal as the mice age (DeLorey *et al.*, 1998). Behavioral observations, coupled with EEG recordings, indicate that these mice are subject to an evolving epileptogenic condition from disorganized electrocortical activity with high amplitude slow and sharp waves to interictal spiking culminating in spontaneous seizures as they mature (DeLorey *et al.*, 1998; Homanics *et al.*, 1997). These observations plausibly suggest, but do not prove, that seizure activity evolves as a self-propelled process engendered by the lack of gabrb3 gene expression at an earlier critical period of development. As discussed earlier, gabrb3 null mice exhibit a reduction in sIPSC in the RTN,

which is associated with the generation of absence type seizures (Huguenard and Prince, 1994).

The incidence of epilepsy in ASD has been reported to be about 33% (Tuchman *et al.*, 1991), however, it is important to note that between 43–68% of ASD individuals exhibit epileptiform EEG activity in sleep without actually manifesting clinical seizures (Chez *et al.*, 2004). Interestingly, when one views separately ASD cases attributed to chromosome 15q11–13 duplications (isodicentric chr 15), the incidence of epilepsy rises to about 71% (Mann *et al.*, 2004; Wolpert *et al.*, 2000), consistent with the involvement of a GABAergic mechanism. ASD and AS patients are frequently described as having EEG paroxysmal abnormalities, multiple seizure types, and an age-dependent evolution of the epilepsy (Kumada *et al.*, 2005; Matsumoto *et al.*, 1992; Rossi *et al.*, 1995). The prevalence rate for seizures in AS is >80% (Minassian *et al.*, 1998; Moncla *et al.*, 1999) with AS patients possessing the large chromosome 15q11–13 region deletion typically exhibiting the more severe seizure phenotype, which is usually harder to treat than the seizures associated with AS patients that just have a point mutation in the UBE3A gene (Minassian *et al.*, 1998). Interestingly, only 29% of the mice with a homozygous disruption of the ube3a gene on the same mixed hybrid background as the gabrb3 null mice exhibit seizures and require audiogenic-induction (Jiang *et al.*, 1998). This is in contrast to the 100% of gabrb3 null mice that exhibit spontaneous seizures (DeLorey *et al.*, 1998). The previous observations in both AS patients and the mouse models suggest a contributing role for the gabrb3 gene in the more robust epileptic phenotype observed in AS deletion patients. Moreover, a revealing case involved a patient that had an unusual maternal 15q deletion that eliminated the $GABA_A$ receptor subunit gene cluster but not the UBE3A and ATP10C genes as usually occurs in AS. Although this individual exhibited moderate mental retardation (IQ 50) he did not meet the full AS diagnostic criteria. However, this patient exhibited a strikingly similar electrocortical disturbance to those seen during sleep in AS patients with large deletions (Michaelis *et al.*, 1995), again implicating the genes in the $GABA_A$ receptor gene cluster (GABRB3, GABRA5, and/or GABRG3) in the electrocortical disturbances that likely contribute to the seizures often associated with AS. The high-amplitude polymorphic slow wave activity seen in EEG of both AS children and gabrb3 null mice, instead of the normally expected alpha and beta frequencies, is reminiscent of the EEG patterns associated with disturbances of cortical-thalamic and cortical-cortical physiology.

B. Antiepileptic Drugs

A variety of antiepileptic drugs (AED) have been administered to gabrb3 null mice, with ethosuximide being the most potent in lessening seizures and normalizing EEG abnormalities (DeLorey *et al.*, 1998). Ethosuximide is generally used

for the control of absence seizures and works by inhibiting T-type calcium channels involved in synchronization of thalamocortical circuitry (Huguenard, 1999). Carbamazepine was found to worsen seizures and EEG in gabrb3 null mice (DeLorey *et al.*, 1998).

Anticonvulsants that have been most often reported as being beneficial in treating seizures in AS include valproic acid and benzodiazepines, with ethosuximide and topiramate also receiving favorable results (Franz *et al.*, 2000; Laan *et al.*, 1996; Ostergaard and Balslev, 2001; Sugiura *et al.*, 2001). Carbamazepine, oxcarbazepine, and vigabatrin have been reported to exacerbate seizures in both frequency and severity in AS (Guerrini *et al.*, 2003; Minassian *et al.*, 1998; Ostergaard and Balslev, 2001). Interestingly, lamotrigine, which has no direct effect on $GABA_A$ receptors but has been shown to increase the expression of the GABRB3 gene, is also considered effective in the treatment of the epilepsy associated with AS (Gibbs *et al.*, 2002; Ruggieri and McShane, 1998; Wang *et al.*, 2002). Few controlled clinical trials of AEDs in the treatment of ASD associated epilepsy have been conducted, likely due to the high heterogeneity observed in this disorder. However, one study reported that carbamazepine triggered new-onset epileptic seizures in an individual with ASD (Monji *et al.*, 2004). Interestingly, lamotrigine was found to improve autistic symptoms in 8/13 ASD children, separate of whether there was an improvement in their epilepsy or not (Uvebrant and Bauziene, 1994).

VII. Behavior

A. Social Behavior

To date there have been no reports of the social characteristics of the gabrb3 null mouse, other than the noted failure of dams to display appropriate nurturing behavior toward their offspring, irrespective of the genotype of the offspring (Homanics *et al.*, 1997). Impaired social behavior is a core feature of ASD (Gillberg *et al.*, 1996) and has also been reported in AS (Penner *et al.*, 1993). Efforts to establish whether social parameters are altered in gabrb3 null mouse have become of paramount importance and are currently underway.

B. Stereotypical Behavior

Gabrb3 null mice are easily excited, which elicits hyperactive behavior in the gabrb3 null mice, during which time they often display repetitive peripheral circling or tight turning in place, as if they were chasing their own tails (Homanics

et al., 1997). This type of behavior has likewise been observed in mice with extensive Purkinje cell loss in the cerebellum (Fransen *et al.*, 1998). Interestingly, recent studies in rat have implicated GABAergic mechanisms in the substantia nigra pars reticulata working in relationship with the dopaminergic system in the substantia nigra pars compacta as contributing to circling behavior (Velisek *et al.*, 2005).

A diagnostic criteria of both ASD and AS is stereotypical behavior, in which affected individuals exhibit motor responses that are repetitive, invariant, and seemingly without purpose or goal (Barthelemy *et al.*, 1997; Summers *et al.*, 1995). Interestingly, a circling (spinning) behavior is often reported in individuals with ASD (Bracha *et al.*, 1995).

C. Motor Coordination and Locomotor Activity

The cerebellum is one of the first brain structures to begin to differentiate, yet it is one of the last to achieve maturity; its cellular organization continues to change well after birth. During this transitional period a strong correlation exists between the high expression of β_3 subunit message and the formation of multiple connections between the inferior olive climbing fibers (axons) and Purkinje cells (Frostholm *et al.*, 1992). This protracted developmental process creates a special susceptibility to disruptions during development; such disruptions would likely reveal themselves through changes in motor coordination and locomotor abilities. When held by the tail gabrb3 null mice tend to ball-up by clasping their paws together rather than splaying their paws outwards as is observed in wildtype mice and the progenitor strains used in creating the gabrb3 null mice; this trait is often associated with neurological impairment (Homanics *et al.*, 1997). In addition, gabrb3 null mice have difficulty swimming, walking on wire grid floors, repeatedly fall off platforms, and perform poorly on the accelerating rota-rod task (DeLorey *et al.*, 1998; Homanics *et al.*, 1997). Similarly, homozygous p^{cp} mice, which lack the $GABA_A$ receptor subunit genes, gabra5 and gabrg3 in addition to the gabrb3 gene, are described as being ataxic (Nakatsu *et al.*, 1993). Gabrb3 null mice are also hyperactive as compared to their wildtype littermates in measures of cage crossings and velocity and are easily discerned from their wildtype littermates by casual observation (DeLorey *et al.*, 1998). This study also found gabrb3 null mice to be strikingly different from wildtype mice in both duration of their active period and overall total activity when monitored over a 3-day period (DeLorey *et al.*, 1998).

AS patients exhibit strong motor disturbances including ataxia and poor motor control (Williams *et al.*, 1995). ASD individuals likewise exhibit deficits in complex or skilled motor movements and are often described as being clumsy (Minshew *et al.*, 1997) with some individuals being described as having "a bizarre

gait" (Barthelemy *et al.*, 1997). In addition, there are reports that the achievement of motor milestones in the developing autistic child is delayed (Teitelbaum *et al.*, 1998). Lastly, a behavioral hallmark often reported in both ASD and AS is hyperactivity as well as easy excitability (Barthelemy *et al.*, 1997; Summers *et al.*, 1995; Williams *et al.*, 1995).

D. Hyperresponsivity

One of the earliest traits noted in gabrb3 null mice was that they were hyperresponsive to being handled or exposed to other sensory stimuli, which usually culminated in the expression of hyperactive behavior and stereotypical circling mentioned earlier (Homanics *et al.*, 1997). Gabrb3 null mice were found to display enhanced responsiveness, compared to wildtype mice, to low-intensity thermal stimuli in the tail-flick and hot-plate tests (Ugarte *et al.*, 2000). In addition, the same study reported that the gabrb3 null mice exhibited enhanced responsiveness to innocuous tactile stimuli compared to wildtype mice as assessed with von Frey filaments. They suggested that the occurrence of such post-stimulus behaviors as vocalization, fending behavior, and licking or shaking of hindpaws suggests that the decrease in mechanical thresholds is a sensory effect rather than a secondary facilitated motor response. The presence of thermal hyperalgesia and tactile allodynia in the gabrb3 null mice is consistent with a loss of inhibition of somatosensory transmission mediated by presynaptic and post-synaptic $GABA_A$ receptors in the spinal cord where the majority of $GABA_A$ receptors express mRNA for the β_3 subunit (Ma *et al.*, 1993; Persohn *et al.*, 1991; Zhang *et al.*, 1991). A wealth of evidence indicates that GABA and $GABA_A$ receptors in the spinal cord, medulla, and pons play important roles in the modulations of nociception (Hammond, 1997). The previous results are also consistent with electrophysiological investigations in which $GABA_A$ receptor antagonists were applied to the spinal cord resulting in an enhanced response to light touch, further supporting a role for GABA and $GABA_A$ receptors in somatosensation (Sivilotti and Woolf, 1994). The previous findings likewise implicate $GABA_A$ receptors in the tonic inhibition of low threshold afferent inputs to the spinal cord and also suggest high threshold thermoreceptive inputs to the spinal cord are tonically inhibited to a lesser extent.

A sensory profile of 26 children with ASD (age range 2–3.5 yrs) and 24 normal children (age range 1–3 yrs), found the ASD children to exhibit significantly heightened tactile sensitivity compared to the normal children (Rogers *et al.*, 2003). Other studies likewise reported ASD children to exhibit hyperresponsivity to touch (Ayres and Tickle, 1980) and tactile defensiveness (Baranek *et al.*, 1997). In addition, in a study of 25 ASD individuals, 44% were reported to exhibit hypo/hypersensitivity to temperature (Harrison and Hare, 2004). Neither

tactile defensiveness or heat sensitivity has been investigated in AS, however, parents often anecdotally report their AS child as exhibiting tactile defensiveness and/or hypersensitivity to heat.

E. Sleep Architecture

Assessment of sleep states and sleep electroencephalography in gabrb3 null mice revealed little difference from wildtype mice in non-rapid eye movement sleep (NREMS) time but found substantial differences between the two genotypes in regards to the EEG spectral characteristics during NREM sleep (Wisor *et al.*, 2002). In addition, gabrb3 null mice exhibited significantly less REM sleep time compared to wildtype mice measured during the light portion of a 24-hr light-dark cycle (Wisor *et al.*, 2002). This finding is in line with studies supporting a critical role for GABAergic transmission in the regulation of REM sleep (Rye, 1997), thereby implicating the β_3 subunit of the $GABA_A$ receptor in the regulation of the cortical expression of sleep states. The observed differences seen in the gabrb3 null mice are likely the result of the disrupted RTN allowing hypersynchronous activity to occur in the thalamic circuitry, discussed earlier, which regulates cortical expression of NREMS. Significant differences in EEG spectral power during the transition from wake-NREMS were also noted between the two genotypes. In addition, the transient increase in EEG power in the 12–16 Hz range that occurs in wildtype mice during the transition from NREM to REM sleep was significantly blunted in the gabrb3 null mice. The ramifications of this finding are unclear, however sleep spindles, transient 11–16 Hz oscillations occurring in the cortical EEG and are prominent during the transition from NREMS to REMS, are generated by the RTN (Destexhe *et al.*, 1998; McCormick and Bal, 1997) and are impaired in the gabrb3 null mice as discussed earlier. Interestingly, circadian rhythms monitored over a nine-day period in the mutant mice appear to remain intact. The previous findings clearly indicate that disrupting the gabrb3 gene in these mice leads to significant changes in normal sleep architecture.

Sleep disturbances and atypical sleep architecture are frequently reported in both ASD (Limoges *et al.*, 2005) and AS (Bruni *et al.*, 2004; Clayton-Smith, 1993; Miano *et al.*, 2004). Likewise, AS patients of 10 years of age or younger exhibit similar sleep disturbances to those characterized in ASD including problems with initiating sleep with long latencies to fall asleep, excessive nocturnal awakenings, reduced hours of sleep per night, poor sleep quality, unusual and jerky movements during sleep, and daytime sleepiness (Bruni *et al.*, 2004). A reduction in REM sleep time has also been reported in AS individuals compared to controls (Miano *et al.*, 2004). ASD individuals (age range: 16–27 years) also exhibit longer latencies to fall asleep, frequent nocturnal awakenings, lower sleep efficiency and

quality, more daytime sleepiness, decreased non-REM sleep, less sleep spindles during stage 2 sleep, and a lower number of rapid eye movements during REM sleep (Limoges *et al.*, 2005).

F. Learning Deficits

Gabrb3 null mice display a deficiency in Pavlovian fear conditioned contextual memory compared to wildtype littermates (DeLorey *et al.*, 1998). This type of conditioning is a rapidly acquired form of learning thought to be a model of human explicit memory that is dependent on the induction of long-term potentiation in the hippocampus (Kim and Fanselow, 1992) and the functional integrity of the cerebellar vermis (Sacchetti *et al.*, 2002). Both the hippocampus and the cerebellum have been implicated in ASD (Bauman and Kemper, 1985) and contain abundant amounts of the β_3 subunit during development and in adulthood. Gabrb3 null mice also exhibited defective operant learning as measured in the passive avoidance task (DeLorey *et al.*, 1998), which has likewise been suggested to involve the hippocampus (Lorenzini *et al.*, 1996).

A prominent feature of ASD and AS is an association with mental retardation (Edwards and Bristol, 1991; Williams *et al.*, 1995) with reports of autistic individuals exhibiting impairments in spatial working memory (Minshew *et al.*, 1999).

VIII. Concluding Remarks

The growing evidence implicating GABAergic mechanisms in the etiology of ASD and AS, mirrored by the numerous neurochemical, electrophysiological, and behavioral similarities shared between these disorders and the gabrb3 null mice (Table I), lend support for the involvement of the GABRB3 gene in the etiology of these disorders. Moreover, because ASD is generally believed to be caused by multiple mechanisms, it may be worthwhile to also consider that the co-inheritance of a combination of hypomorphic alleles, each affecting overall GABAergic tone, could likewise be the basis of some forms of ASD. As GABAergic neurons are known to be essential to information processing in almost every brain region, one would expect that a reduction in GABAergic function, of any nature, would result in these regions becoming hyper-excitable, consequently impairing neural information processing, a feature considered to be central to ASD (Belmonte *et al.*, 2004). Gabrb3 gene deficient mice, as well as other animal models of GABAergic dysfunction, remain valuable tools in the critical examination of the interconnection between development, pathology, and behavior as they relate to disorders of neurodevelopment.

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THE REELER MOUSE: ANATOMY OF A MUTANT

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The neurological mouse mutant *reeler* is characterized by ataxia and disruption of cellular layers in the brain. This mutant has long been studied as a model to understand how cortical structures of the vertebrate brain are formed during development. The *reeler* phenotype arises from homozygous loss-of-function mutations in *reelin*, a gene that encodes an extracellular glycoprotein secreted by distinct neuronal populations during embryonic and postnatal brain development. Reelin is a key regulator of cortical development that appears to function as a switch, causing neurons to terminate their migration phase and to begin the assembly of cortical layers. The Reelin signal is interpreted by neurons and neuronal progenitors through signal transducing molecules, such as the VLDLR and the ApoER2 receptors, and the Dab1 adapter protein. Loss of these essential transducers in mutant mice results in the appearance of a phenotype indistinguishable from *reeler*. Much has been learned during the past few years about the molecular mechanisms that mediate the Reelin signal. This large protein is

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thought to cluster the VLDLR and ApoER2 receptors, thereby activating src-family kinases that phosphorylate Dab1 on tyrosine residues. This event in turn causes Dab1 to interact with a variety of signal transduction molecules and proteins that regulate cytoskeleton dynamics, before being degraded by the proteosome pathway. Expression of Reelin and its transduction machinery continues long after migration is complete and may affect neuronal maturation and synaptic connectivity in the postnatal brain. Despite the tremendous progress made in this past decade, the whole spectrum of Reelin activities in brain development and its relevance to human cognitive disorders has yet to be fully unraveled.

I. Introduction

Reeler is a well-characterized autosomal mutation in the mouse that affects several aspects of brain development. The first known *reeler* mutation occurred spontaneously in 1948, in an inbred mouse colony in Edinburgh, Scotland, and was thus referred to as rl^{Ed}. Mutant mice, which appeared in this strain at the frequency expected for a recessive trait according to classical Mendelian inheritance, were severely ataxic and exhibited a characteristic reeling gait that conferred them their name. Anatomical studies indicated that all major cortical structures of the brain were present, but appeared disorganized in *reeler* mutants. Strikingly, projection neurons were shown to connect properly to their ectopic targets. For example, thalamocortical projections terminated properly on neurons destined for cortical layer IV, even though these occupied abnormal positions in the *reeler* cortex (Molnar *et al.*, 1998; Steindler and Colwell, 1976). Climbing fibers also terminate properly on ectopically positioned Purkinje cells in the *reeler* cerebellum, although subtle defects in the number and position of their synaptic boutons are present (Mariani, 1982). The observation that all major neuronal types are born at the correct time in the *reeler* brain, but end up in the wrong location led to the realization that the study of the *reeler* mouse could provide exquisite insights into the mechanisms of neuronal migration and cortical layer formation (reviewed by Caviness and Rakic, 1978; D'Arcangelo and Curran, 1998; Goffinet, 1984b; Lambert de Rouvroit and Goffinet, 1998). Indeed, an entirely novel signaling pathway was discovered following the identification in 1995 of the *reeler* gene, which was called *reelin* (D'Arcangelo *et al.*, 1995). This gene is mutated not only in the original rl^{Ed} strain, now designated $Reln^{rl}$ and commercially available from The Jackson Laboratory, but also in several other *reeler* mouse strains. All of the known *reeler* strains lack functional Reelin protein and are therefore null (Andersen *et al.*, 2002; D'Arcangelo and Curran, 1998). Most strains lack *reelin* mRNA expression altogether, due to large deletions

of the gene or exon skipping that renders the transcript highly unstable. The Orleans strain (rl^{Orl}) is unique in that a small 3' deletion in the coding sequence, due to a retroviral insertion, gives origin to a Reelin protein that lacks the C terminus and cannot be secreted (D'Arcangelo *et al.*, 1997; de Bergeyck *et al.*, 1997). The phenotype among all *reeler* strains is essentially the same. Recently, two *reelin*-deficient rat strains have been described (Kikkawa *et al.*, 2003; Yokoi *et al.*, 2003). The Shaking Rat Kawasaki (SRK) and the Komeda Zucker Creeping (KZC) rats display all of the typical anatomical features of the mouse *reeler* mutation. These animals may be of particular use for future behavioral and pharmacological studies, for which the rat is the preferred species.

This chapter will describe the most salient anatomical aspects of the *reeler* mutation, I will then recapitulate the key molecular discoveries and finally attempt to integrate these findings into a framework that explains how Reelin may regulate brain development and function.

II. Neuroanatomy of *Reeler* and *Reeler*-Like Mutants

A. Neocortical Development in *Reeler*

The development of the neocortex in *reeler* has been previously reviewed in detail (Caviness and Rakic, 1978; Caviness *et al.*, 1988; Goffinet, 1979, 1984b). However, because some general concepts in neurogenesis and migration have evolved over the past few years I will briefly revisit the issue here (see Fig. 1). In the early embryonic brain, the cell bodies of neuroepithelial cells (radial glia) reside near the ventricle and extend cellular processes towards both the ventricular and the pial surface. These cells were for a long time considered postmitotic glia whose function was solely to provide support for neuronal migration, but it is now clear that radial cells are dividing progenitor cells that produce both neurons and glia, depending on the developmental stage of the cortex (Malatesta *et al.*, 2000, 2003; Noctor *et al.*, 2001, 2004). The first postmitotic neurons in the cortex, the pioneer neurons, appear superficially to the germinal layer and form a transient structure named the preplate (Marin-Padilla, 1978; Meyer *et al.*, 1998). This structure contains two transient neuronal populations, the Cajal-Retzius cells (located superficially) and the subplate cells (located deeply). Cajal-Retzius cells express high levels of Reelin throughout corticogenesis and are thus essential for normal brain development. It is now believed that these early neurons originate from extracortical sites and migrate into the neocortex by tangential migration (Takiguchi-Hayashi *et al.*, 2004). Subplate cells, on the other hand, are important for targeting of thalamocortical afferents into the early neocortex (Shatz *et al.*, 1990). Cortical neurons destined to become excitatory

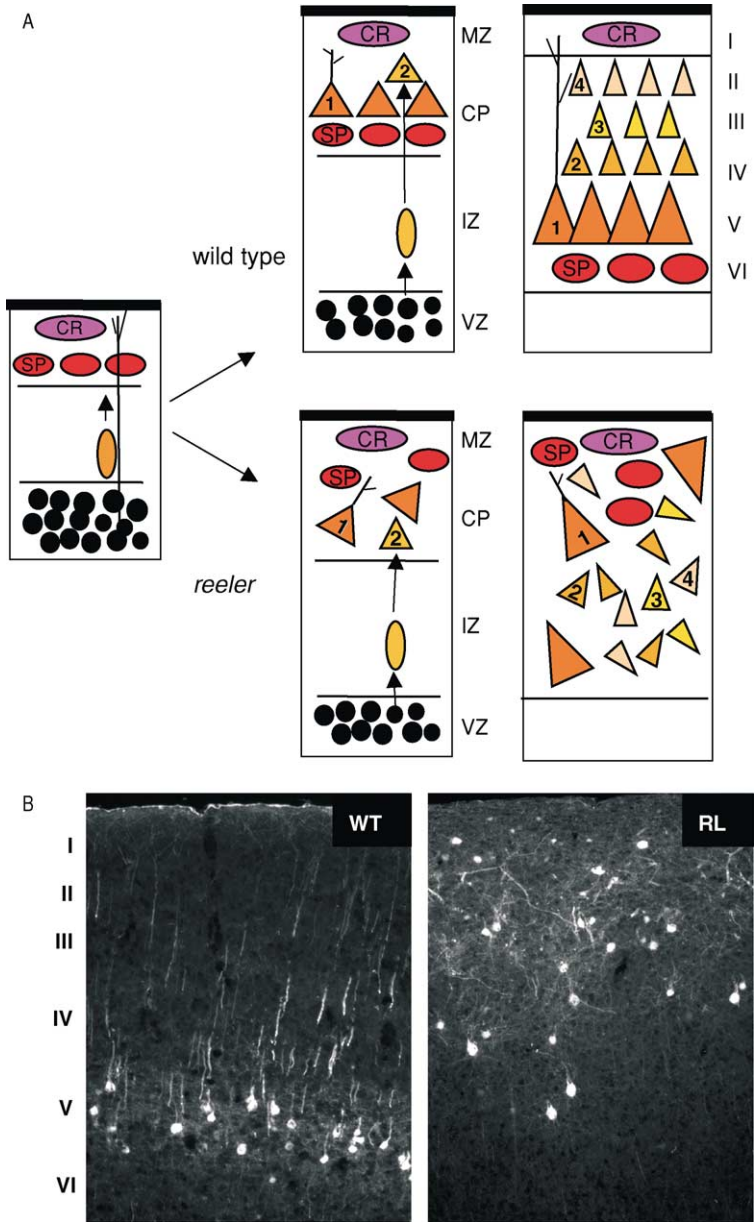

FIG. 1. A. Diagram of neocortical development in wild type and *reeler* mice. Principal neurons are born in the ventricular zone (VZ) and migrate radially toward the marginal zone (MZ). In wild type cortex, the first-born principal neurons (labeled 1) split the preplate composed of Reelin-expressing Cajal-Retzius (CR) and subplate cells (SP). Each wave of later-born neurons (labeled sequentially with

(principal neurons) are derived from radial progenitors in the germinal layer of the cortex. These neurons migrate radially toward the pial surface along the process of the mother radial cell, and split the preplate by positioning themselves between Cajal-Retzius and subplate cells (Marin-Padilla, 1978). Birthdating studies demonstrated that newborn principal neurons bypass their predecessors and terminate just beneath Reelin-rich Cajal-Retzius cells according to an inside-out patterning (Angevine and Sidman, 1961). Inhibitory cortical neurons (interneurons), on the other hand, are generated in subpallial areas and migrate into the developing neocortex by tangential migration, independently of radial guidance (reviewed in Marin and Rubenstein, 2001). Once they enter the neocortex, interneurons also move radially to terminate in specific layers, depending on their subtype, following cues provided by principal neurons or other laminar determinants.

From an examination of the anatomy of the *reeler* cortex, it is apparent that this mutation specifically alters the position of all neurons in the radial dimension of the cortex (Fig. 1). The preplate forms normally in *reeler*, but principal neurons fail to split it and instead accumulate underneath subplate cells. Each subsequent neuronal cohort fails to penetrate the preplate and to bypass older neurons. Instead of forming cellular layers, neurons either spread throughout the mutant cortex or become ectopically positioned in the wrong layer, giving rise to approximately inverted layers. These layers are very abnormal because they contain neurons that not only are born at a time that is inappropriate for the position they occupy, but also because they are grossly disoriented. *Reeler* neurons fail to acquire or maintain a proper radial orientation, and also maintain an abnormally tight association with the radial process of germinal cells. This observation led to the 'obstructed migration' hypothesis, which postulates that earlier principal neurons in *reeler* get stuck on radial fibers and thus prevent older neurons from moving forward (Pinto-Lord *et al.*, 1982). Interneurons also appear disoriented and ectopic in the *reeler* cortex, although it is not clear whether this is a secondary consequence of the principal neuron ectopia (Hevner *et al.*, 2004; Yabut *et al.*, in press). Another aspect of the *reeler* cortical defect is that the radial fiber scaffold is abnormal. Unlike the normal cortex where radial fibers terminate and branch in the Reelin–rich marginal zone, in *reeler* the radial fibers are slightly oblique, with end feet that often terminate below the marginal zone (Derer, 1979; Hartfuss *et al.*, 2003). The radial progenitors also disappear prematurely in *reeler*, grating neurons of their guidance (Derer, 1979; Hunter-Schaedle, 1997; Hartfuss

arabic numbers) bypasses their predecessors and forms cortical layers (labeled with roman numbers). In *reeler*, principal neurons do not split the preplate and fail to form layers. CP = cortical plate. IZ = intermediate zone. B. Fluorescence images of layer V neurons labeled with YFP in the adult wild type (WT) and *reeler* (RL) neocortex.

et al., 2003). Whether the *reeler* defects in corticogenesis are due to abnormalities intrinsic to neurons and/or radial cells is still debated. *In vitro* studies indicate that Reelin directly affects the development of both neurons and radial cells and that both cell types express Reelin signaling proteins (Hartfuss *et al.*, 2003; Niu *et al.*, 2004). The contribution of each cell type to the *reeler* defect may vary in different regions of the brain and is not completely elucidated (see later discussion).

B. Hippocampal Development in *Reeler*

In the hippocampal formation the development of the hippocampus proper and that of the dentate gyrus proceed according to their own distinct morphogenetic patterns (see Fig. 2). In the hippocampus proper, principal neurons destined

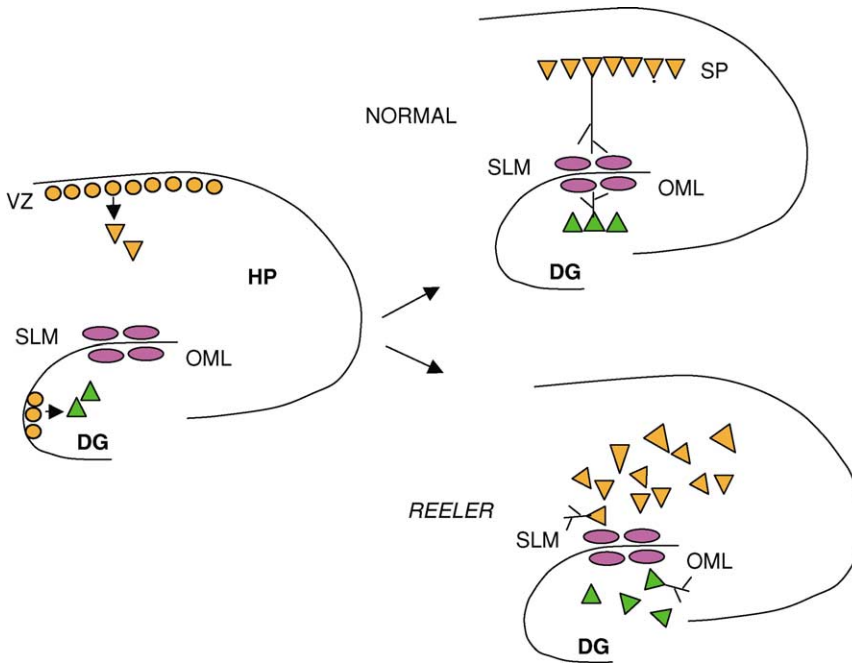

Fig. 2. Diagram of hippocampal development in normal and *reeler* mice. In the hippocampus proper (HP) pyramidal neurons are born in the ventricular zone (VZ) and migrate radially towards the stratum lacunosum moleculare (SLM), which contains Reelin-expressing Cajal-Retzius cells (large ovals). In normal mice they form a distinct pyramidal layer and project apical dendrites to the SLM. In the dentate gyrus (DG), granule neurons migrate inwardly from the outer molecular layer (OML) to form a compact layer. The OML also contains Reelin-expressing Cajal-Retzius cells. In *reeler* both pyramidal and granule cell layers fail to form.

for the pyramidal layer migrate radially from the ventricular zone toward the stratum lacunosum moleculare (near the pial surface) where Reelin-expressing Cajal-Retzius cells reside, similarly to principal neurons in the neocortex. Unlike their neocortical counterparts, however, hippocampal neurons terminate their migration well before they reach the pial surface and accumulate according to an inside-out pattern in the pyramidal layer. In *reeler* these neurons fail to assemble in a tight layer and become disoriented and distributed throughout the hippocampus proper (Fig. 2).

In the dentate gyrus, granule neurons born mostly postnatally in a superficial germinal zone migrate inwardly to form a compact layer underneath Reelin-expressing Cajal-Retzius cells in the outer molecular layer. In *reeler* these neurons fail to form a distinct cellular layer and are grossly disoriented, with their dendrites often appearing oblique to the radial scaffold. In addition to malposition, a dramatic reduction in the complexity and the length of dendritic processes of hippocampal neurons has been described both *in vivo* and *in vitro* (Niu *et al.*, 2004; Stanfield and Cowan, 1979). Disorganization of the radial fiber scaffold is also prominently observed (Forster *et al.*, 2002; Weiss *et al.*, 2003; Zhao *et al.*, 2004), unlike the neocortex where this structure is only modestly affected.

An additional, unusual aspect of the *reeler* hippocampal phenotype is the delayed development of the entorhinohippocampal projections (Del Rio *et al.*, 1997). These axons terminate and branch profusely in the Reelin-rich stratum lacunosum moleculare and outer molecular layer of normal mice. In *reeler*, the axons' terminals appear less developed, at least during the first postnatal weeks. As a consequence of this delayed maturation, the formation of synaptic contacts between entorhinohippocampal neurons and their targets is impaired (Borrell *et al.*, 1999). Thus, in the hippocampus, Reelin is required not only for cell body positioning and dendrite elongation, but also for axonal branching and synaptogenesis.

C. Cerebellar Development in *Reeler*

The cerebellum is the brain structure most devastated by the *reeler* mutation and likely responsible for the ataxic gait of the mutant (see Fig. 3). The most obviously disrupted regions of the cerebellum are the hemispheres, which normally consist of several folia, but in *reeler* they are severely reduced. This is a result of a remarkable reduction in cell number, which affects not only the most abundant cerebellar cell type, the granule cells, but also the Purkinje cells, although to a lesser extent (Heckroth *et al.*, 1989). This massive growth deficit is due to a cell layering defect that can be first observed during early embryogenesis (Goffinet, 1983a). In normal mice, Purkinje cells, which are the principal neurons

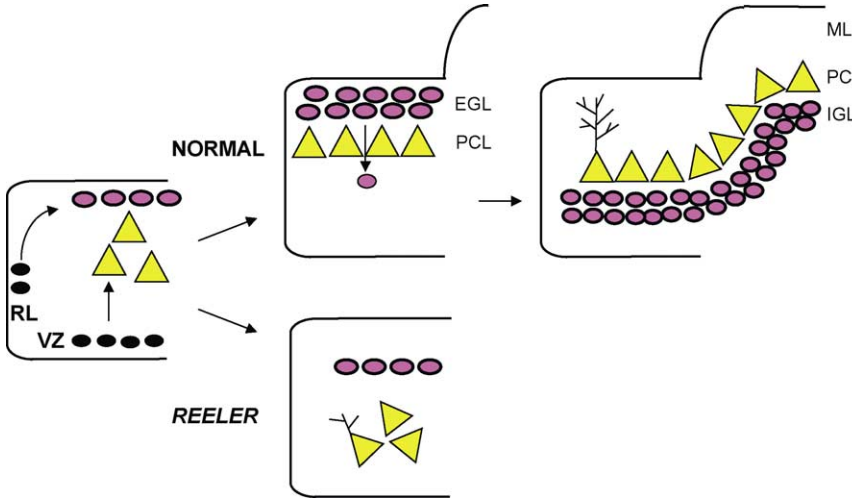

FIG. 3. Diagram of cerebellar development in normal and *reeler* mice. In the cerebellum Reelin-expressing granule cells are born in the rhombic lip (RL) and migrate tangentially to form the external granular layer (EGL). Purkinje cells (triangles) are born in ventricular zone (VZ) and migrate radially toward the EGL. In normal mice Purkinje cells form a layer (PCL). Granule cells proliferate and migrate across the PCL to form the internal granule layer (IGL). The cerebellum contains many folia. In *reeler*, Purkinje cells fail to form a layer and granule cells do not proliferate extensively, leading to reduced foliation.

of the cerebellum, are born in the ventricular layer and migrate radially toward the pia. Along this path, they encounter Reelin-expressing cells derived from the rhombic lip, including cells destined for the granule layer and deep cerebellar nuclei. The granule cells then form a displaced germinal layer, the external granule layer (EGL), superficially to the Purkinje cells. This juxtaposition of Purkinje cells to Reelin-expressing cells during embryogenesis results in the formation of a compact Purkinje cell layer (PCL) underneath the EGL. The PCL is initially multicellular, but is later transformed into a single cell layer by the expansions of the cerebellar cortex during early postnatal ages. In *reeler* the PCL does not form and Purkinje cells remain deep in an amorphic central mass (Goffinet *et al.*, 1984; Mariani *et al.*, 1977; Mikoshiba *et al.*, 1980) (Fig. 3). Their orientation is very abnormal and the development of their dendrite trees is severely impaired, as for principal forebrain neurons. It has been proposed that the layering defect in the cerebellum, similarly to that in the neocortex, is due to obstructed migration of Purkinje cells along radial fibers (Yuasa *et al.*, 1993). An alternate possibility is that Purkinje cells fail to achieve proper radial orientation or to express adhesion molecules required for the formation of a compact cell

layer. After PCL formation, the normal development of the cerebellum continues into the first 1–2 postnatal weeks with the inward migration of granule cells along glial fibers and across the PCL to form an internal granule layer (IGL). This process is obviously disrupted in *reeler*, given the absence of a PCL, and mutant granule cells remain loosely arranged in a cellular layer mostly superficial to ectopic Purkinje cells. Granule cell ectopia is likely to be secondary to the absence of a PCL and not due to a migration defect *per se*. *In vitro* studies have in fact shown that the capacity of *reeler* granule cells to migrate along glial fibers is not impaired (Nagata and Terashima, 1994). The dramatic reduction in granule cell number in *reeler* is thought to arise from the scarcity of Purkinje cell-derived growth factors in the superficial aspects of the cerebellar cortex. However, it is not clear that this is the sole cause of the abnormality, nor is it known what causes the more modest but significant reduction in Purkinje cell number. Thus, other possibilities such as a novel function of the *reelin* gene on cell proliferation or determination cannot presently be discounted.

D. Brain Stem and Spinal Cord Development in *Reeler*

In addition to the cerebellum, some laminated hindbrain structures also appear disrupted in *reeler*. In the brain stem, the facial nerve nucleus (Goffinet, 1984a; Terashima *et al.*, 1993), the mesencephalic trigeminal nucleus (Terashima, 1996), the cochlear nucleus (Martin, 1981), dopaminergic neurons of the substantia nigra (Nishikawa *et al.*, 2003), the inferior olives (Goffinet, 1983b), the nucleus ambiguous (Fujimoto *et al.*, 1998), the olivocochlear efferent neurons, and the facial visceral motor nucleus (Rossel *et al.*, 2005) all appear disorganized. In some cases the phenotype could be interpreted as a failure of recognition and adhesion among similar subtypes of neurons. In the case of the olivocochlear efferent neurons and facial visceral motor neurons, it appears that these neurons specifically fail to complete a second phase of radial-guided migration toward the Reelin-expressing region (Rossel *et al.*, 2005). Similarly, in the *reeler* spinal cord, specific defects occurring in a later migration step cause the malpositioning of autonomic neurons, and sympathetic as well as parasympathetic preganglionic neurons occupy ectopic positions (Kubasak *et al.*, 2004; Phelps *et al.*, 2002; Yip *et al.*, 2000, 2003). Sympathetic preganglionic neurons (SPNs) undergo a normal initial migration toward the ventrolateral aspect of the developing spinal cord, but fail to complete a second dorsolateral migration toward the intermediolateral column, and instead slip back toward the central canal. Since Reelin is expressed by V1 and V2 interneurons located just medially to the SPNs in the ventral spinal cord, it has been suggested that Reelin may act as a physical barrier, keeping the SPNs in place and preventing them from moving back toward the central canal (Yip *et al.*, 2004b). The recent observation that Reelin is actually transported

from the cell bodies of interneurons along cellular process and released at the ventrolateral surface of the spinal cord suggests that, like in cortical structures of the brain, Reelin promotes an alignment of target neurons just beneath the pial surface (Kubasak *et al.*, 2004).

E. Retinal Development in *Reeler*

In the brain structures previously described, cells expressing Reelin during development appear to be strategically positioned to delimit radial migration and to provide a cue for proper orientation. In the retina, however, a different situation occurs. Ganglion cells express Reelin at the superficial edge of the retina during normal development, but there is no obvious disruption of cellular layer formation in the retina of *reeler* mice (Rice *et al.*, 1998). All retinal cell types in *reeler* are positioned in the appropriate layer and present a normal orientation. However, the stratification of AII amacrine cell synapses controlling the ON and OFF response to light stimuli is altered in the mutant. These cells normally establish synaptic contacts with rod and cone bipolar cells in two sublaminae of the inner plexiform layer, which correspond to ON and OFF responses. In the *reeler* retina, AII amacrine cells establish connectivity with their bipolar cell targets in ectopic positions outside the sublaminae of the inner plexiform layer, leading to the death of many rod bipolar cells and the disruption of the circuitry of the retina (Rice *et al.*, 2001). The resulting physiological defect in the *reeler* retina is the disruption of the ON-OFF organization and the attenuation of rod-driven retinal responses. These findings underscore the importance of Reelin not only in the formation of cellular layers, but also in the development of synaptic layers which in turn impacts the functional circuitry of the brain.

III. Reelin

A. The Reelin Protein

The gene responsible for the *reeler* phenotype was cloned a decade ago by taking advantage of a transgenic allele generated by insertional mutagenesis (rl^{tg}) (Miao *et al.*, 1994). The newly named *reelin* cDNA sequence predicted the synthesis of a large, secreted protein of 385 kDa (D'Arcangelo *et al.*, 1995). The encoded Reelin protein consists of an N terminal region containing a canonical signal peptide and a sequence of modest similarity to F-Spondin, followed by a main region containing eight consecutive repeats unique to Reelin, each featuring a cysteine pattern (EGF-like repeat) typically found in extracellular proteins,

and finally a small C terminal region containing highly charged amino acids (see Fig. 4). Putative glycosylation sites were also noted throughout the sequence. Expression studies confirmed that the Reelin protein produced by cells transfected with the cloned cDNA or by wild type neuronal cultures indeed is secreted in the culture medium, and is subjected primarily to N-glycosylation (D'Arcangelo *et al.*, 1997). The Reelin protein was also immunoprecipitated by the monoclonal antibody CR-50, which selectively recognizes an epitope in the N terminal region of Reelin (D'Arcangelo *et al.*, 1997). This antibody was developed concurrently with the cloning of *reelin*, based on its ability to recognize an antigen absent in *reeler*, and to interfere with the aggregation of wild type cortical neurons *in vitro* (Ogawa *et al.*, 1995). The CR-50 epitope corresponds to a native conformation of the N terminus of Reelin and appears to mediate the formation of homodimers, which may be required for optimal activity during corticogenesis (Kubo *et al.*, 2002). Consistent with this hypothesis, the CR-50 antibody was also shown to interfere with Reelin-induced layer formation in cerebellar explants *in vitro*

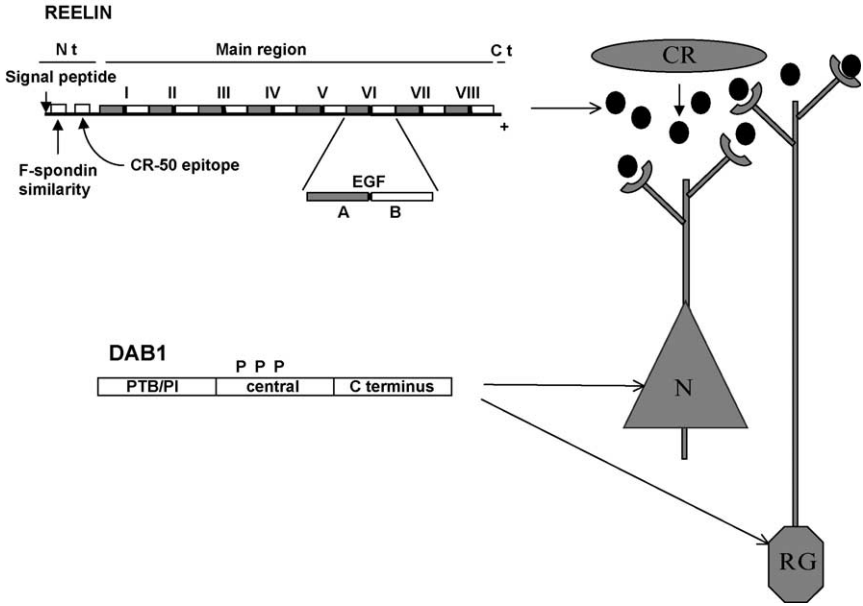

FIG. 4. Cajal-Retzius cells (CR) secrete Reelin (circles) in the extracellular environment of the marginal zone during cortical development. Migrating neurons (N) and radial glia cells (RG) express Reelin receptors and Dab1, and thus receive the Reelin signal on their superficial processes. Schematic representations of the Reelin and Dab1 proteins are shown. Roman numbers indicate Reelin repeats. Reelin-induced tyrosine phosphorylation sites on Dab1 are indicated by Ps. Nt = N terminus. Ct = C terminus.

(Miyata *et al.*, 1997), and in the hippocampus *in vivo* (Nakajima *et al.*, 1997). The C terminal region contains a domain that is necessary for Reelin secretion (D'Arcangelo *et al.*, 1997). Deletion of this domain in the rl^{Orl} strain results in the intracellular accumulation of Reelin and the appearance of a null phenotype (de Bergeyck *et al.*, 1997; Hirotsune *et al.*, 1995).

The initial biochemical studies of Reelin utilized metabolic labeling followed by CR-50 immunoprecipitation, and preferentially detected newly synthesized protein in its full-length form of approximately 400 kDa (D'Arcangelo *et al.*, 1997). Subsequent studies utilized newly developed N terminal Reelin monoclonal antibodies in Western blot assays (de Bergeyck *et al.*, 1998) and identified several proteolytic products, in addition to the full-length protein (D'Arcangelo *et al.*, 1999; de Bergeyck *et al.*, 1998). It is now known that Reelin is processed in the post-Golgi or extracellular environment to produce three major fragments, a ~180 kDa N terminal fragment containing the CR-50 epitope and repeats 1 and 2, a central ~120 kDa fragment containing repeats 3 to 6, and a ~100 kDa C terminal fragment containing repeats 7 and 8 and the secretion signal (Jossin *et al.*, 2003a). Fragments derived from partial cleavage were also detected. Among these, only the central fragment has been shown to be biologically active, although it is not as effective as the full-length protein (Jossin *et al.*, 2004). Similar Reelin fragments can be detected in the cerebral spinal fluid and in the blood serum (Smalheiser *et al.*, 2000), indicating that processing is not unique to the brain environment. At the moment, the significance of this processing is not clear.

B. Reelin Expression

Reelin is abundantly and selectively expressed by Cajal-Retzius cells in the marginal zone of the embryonic cortex and hippocampus (Alcantara *et al.*, 1998; D'Arcangelo *et al.*, 1995; Ogawa *et al.*, 1995) (Fig. 4). Thus, one crucial function for these once mysterious cells is to localize Reelin to the end of the migratory path of principal neurons, providing a positional cue that signals the end of radial migration. Consistent with this role, Cajal-Retzius cells begin to disappear at the end of corticogenesis, resulting in the gradual loss of Reelin from the marginal zone of the rodent cortex and hippocampus. Interestingly, some GABAergic neurons, known as subpial granule cells, occupy the marginal zone in the late embryonic human cortex, in close apposition to Cajal-Retzius cells. These late-arriving but long-lasting Reelin-producing cells may constitute a sustained source of Reelin for prolonged corticogenesis in humans (Meyer and Goffinet, 1998). In all species examined, postnatal development is accompanied by a striking shift in the distribution of Reelin-expressing cells. Many GABAergic interneurons in the postnatal and adult brain express Reelin in all layers of the

cortex and throughout the hippocampus (Alcantara *et al.*, 1998; Pesold *et al.*, 1998). The significance of this new expression pattern is not known, but it may relate to a role of Reelin in neuronal maturation and synaptic activity.

In the cerebellum, Reelin is strongly expressed at embryonic ages by granule cells forming the external granule cell layer, and by prospective deep cerebellar neurons in the nuclear transitory zone. The observation that granule cell loss in the Math-1 knock out mouse only results in a partial disruption of the PCL formation implies that other sources of Reelin significantly contribute to Purkinje cell alignment during embryonic cerebellar development (Jensen *et al.*, 2002). In the postnatal cerebellum, Reelin continues to be expressed by granule cells even after they have migrated across the PCL to form the internal granule layer. It is conceivable that this late expression relates to Purkinje cell dendrite maturation or synaptic connectivity between the axons of the granule cells (parallel fibers) and Purkinje cells dendrites, although this possibility has never been directly investigated.

Another prominent site of Reelin expression is the embryonic and postnatal olfactory bulb. Mitral cells and some periglomerular neurons in the lamina granularis externa have been shown to express Reelin (Alcantara *et al.*, 1998). One possible function of Reelin in this structure is to promote the detachment of olfactory neurons from the rostromigratory stream, thus facilitating their entry into the bulb (Hack *et al.*, 2002). However, another possibility is that Reelin may play a role in the refinement of connectivity and synaptic function in the olfactory glomeruli, which contain the apical dendrites of mitral cells and are sites of considerable synaptic plasticity even in the adult brain.

In addition to those mentioned here, many other structures of the central nervous system contain Reelin-expressing cells at some point during development or in the adult (see Alcantara *et al.*, 1998; Ikeda and Terashima, 1997; Schiffmann *et al.*, 1997, for a detailed description). In general terms, it appears that Reelin controls neuronal migration during embryonic development and modulates neuronal maturation and synaptic function postnatally. Indeed, many axonal tracts have also been shown to contain Reelin immunoreactivity, and Reelin may be secreted at the synaptic terminals (Martinez-Cerdeno *et al.*, 2003). Electron microscopy studies have also demonstrated the accumulation of Reelin in postsynaptic spine densities of the forebrain (Pappas *et al.*, 2001; Roberts *et al.*, 2005; Rodriguez *et al.*, 2000).

Reelin expression is not limited to the nervous system, but can be found in several other organs including the dentine pulp (Maurin *et al.*, 2004), liver, and blood (Ikeda and Terashima, 1997; Smalheiser *et al.*, 2000). The role of Reelin in these latter organs is completely unknown. In odontoblasts, Reelin may facilitate the innervation of the dentin-pulp complex by promoting adhesion to the dental nerve endings (Maurin *et al.*, 2004).

IV. The Reelin Signaling Pathway

A. Reelin Receptors

The analysis of the anatomical defects in *reeler* cortical structures implied that radially-migrating neurons in the neocortex, pyramidal neurons of the hippocampus, and Purkinje cells in the cerebellum were all direct or indirect Reelin targets. The identification of the molecular machinery that transduces the Reelin signal in these regions enabled us to determine that these principal neurons are indeed direct targets of Reelin. Genetic and biochemical studies identified two Reelin receptors, both of which are proteins previously thought to function in lipoprotein metabolism: the very low-density lipoprotein receptor (VLDLR) and the apolipoprotein E receptor 2 (ApoER2) (D'Arcangelo *et al.*, 1999; Hiesberger *et al.*, 1999; Trommsdorff *et al.*, 1999). Double knock out mice lacking both these receptors exhibit a *reeler*-like phenotype, demonstrating that they are essential for Reelin function (Trommsdorff *et al.*, 1999). Individual receptor knock out mice do not exhibit an overt behavioral phenotype, but layering defects can be observed especially in the neocortex and hippocampus of ApoER2 mutant mice (Benhayon *et al.*, 2003; Trommsdorff *et al.*, 1999). Together with expression data showing that ApoER2 is more abundantly expressed than VLDLR, genetic data suggest that ApoER2 may be the predominant Reelin receptor, at least in the forebrain. Biochemical studies demonstrated that both ApoER2 and VLDLR bind full-length Reelin with high affinities. Binding of Reelin proteolytic fragments has also been observed, suggesting that proteolysis may not necessarily downregulate Reelin function (Benhayon *et al.*, 2003; D'Arcangelo *et al.*, 1999; Jossin *et al.*, 2004). On the intracellular side, ApoER2 and VLDLR bind the Reelin signaling protein Disabled-1 (Dab1, discussed later) through their NPxY, a motif present in many lipoprotein receptors and other transmembrane proteins that is important for ligand internalization. Similarly to other members of the lipoprotein receptor family, ApoER2 and VLDLR internalize Reelin (D'Arcangelo *et al.*, 1999; Morimura *et al.*, 2005), which thus triggers activation of a tyrosine kinase signaling pathway in the target cell. Recent studies demonstrated that Reelin promotes clustering of lipoprotein receptors and that this event is sufficient to activate the signaling cascade and to induce long-term potentiation (LTP) in hippocampal slices. Cross-linking antibodies directed toward the extracellular domain of the VLDLR and ApoER2 mimic the effect of full-length Reelin treatment on signal transduction and LTP induction (Strasser *et al.*, 2004; Weeber *et al.*, 2002), but are unable to rescue the *reeler* defect in cortical slices *in vitro* (Jossin *et al.*, 2004). This suggests that molecular events other than receptor clustering are important for the full biological activity of Reelin.

B. Disabled-1

The transduction of the Reelin signal necessitates the presence of Disabled-1 (Dab1), an adapter protein capable of binding to the Reelin receptors VLDLR and ApoER2 (Trommsdorff *et al.*, 1999), as well as other NPxY-containing transmembrane proteins such as amyloid precursor protein (APP) (Homayouni *et al.*, 1999; Howell *et al.*, 1999b; Trommsdorff *et al.*, 1998) and integrins (Schmid *et al.*, 2005). Dab1 was originally discovered as a Src-interacting brain protein that is phosphorylated on tyrosine residues in a developmentally regulated fashion (Howell *et al.*, 1997a). Disruption of the *dab1* gene in mice by homologous recombination, or substitution of five predicted src-inducible tyrosine residues results in a *reeler*-like phenotype (Howell *et al.*, 1997b, 2000). Similarly, the disruption of the *dab1* gene by spontaneous or random mutation in the *scrambler* (scm) and *yotari* (yot) mutant mice also produces a phenotype indistinguishable from *reeler* (Sheldon *et al.*, 1997; Ware *et al.*, 1997; Yoneshima *et al.*, 1997).

Dab1 expression is mostly complementary to that of Reelin in developing brain structures, and unequivocally marks the populations of Reelin-responsive cells (Rice *et al.*, 1998). In the embryonic cortex, Dab1 is highly expressed by cortical plate neurons, but is also expressed together with ApoER2 in the ventricular and subventricular zones by progenitor cells and newborn neurons (Luque *et al.*, 2003; Meyer *et al.*, 2003; Perez-Garcia *et al.*, 2004; Rice *et al.*, 1998; Trommsdorff *et al.*, 1999) (Fig. 4). High levels of Dab1 and ApoER2 are also observed in many hippocampal cell populations including pyramidal cells and granule cells of the dentate gyrus. In contrast, expression of Dab1, VLDLR, and ApoER2 in the cerebellum is restricted to the Purkinje cell layer. A limited population of Dab1 and ApoER2/VLDLR-expressing neurons is also observed in the spinal cord, consisting of autonomic preganglionic neurons (Carroll *et al.*, 2001; Phelps *et al.*, 2002; Yip *et al.*, 2004a). Virtually all these cells are ectopic in mice lacking Reelin, ApoER2/VLDLR, or Dab1, demonstrating the essential role of these proteins in Reelin signaling.

The Dab1 protein consists of three functionally distinct domains, an N terminal phosphotyrosine binding and phosphoinositide interacting domain (PTB/PI), a central region containing Reelin-dependent tyrosine residues, and a C terminal domain (Fig. 4). Dab1 binds to the cytoplasmic tail of NPxY-containing transmembrane proteins by virtue of its PTB/PI domain. This domain also binds phosphoinositide (PI) 4,5P_2, which strongly directs its localization to the plasma membrane. Binding to the PI occurs through a different protein interface, indicating that Dab1 most likely binds simultaneously to membrane proteins and lipids (Stolt *et al.*, 2003; Yun *et al.*, 2003). A recent study demonstrated that interaction with both the receptor tail and PI is necessary for Reelin-induced Dab1 phosphorylation (Stolt *et al.*, 2005). This event is linked to the activation of signal transduction pathways as well as the downregulation of

Dab1 expression in target cells. Addition of recombinant Reelin to cultured cortical neurons causes an acute increase in phosphorylation on tyrosine residues 198, 220, and 232 in the central domain of Dab1 (Ballif *et al.*, 2004; Howell *et al.*, 1999a; Keshvara *et al.*, 2001). This induction does not occur in double ApoER2/VLDLR mutant neurons, and it is reduced in single ApoER2 mutant neurons, further indicating that Reelin signaling is mediated largely by ApoER2 in cortical neurons (Benhayon *et al.*, 2003; Trommsdorff *et al.*, 1999). Reelin induction of Dab1 phosphorylation is carried out by the Src family of kinases (SFK) (Arnaud *et al.*, 2003b; Bock and Herz, 2003). Among these, Fyn appears to be the principal kinase, since Dab1 phosphorylation is significantly reduced in Fyn mutant mice (Arnaud *et al.*, 2003b). SFK activation in turn is dependent on the presence of Dab1, suggesting a positive feedback mechanism (Bock and Herz, 2003). Dab1 phosphorylation leads to its ubiquitination and degradation by the proteosome pathway, thus resulting in the downregulation of Reelin signaling (Arnaud *et al.*, 2003a; Suetsugu *et al.*, 2004). These findings explain why Dab1 protein accumulates in *reeler*, double ApoER2/VLDLR, and Fyn mutant mice, in which Dab1 tyrosine phosphorylation is reduced (Arnaud *et al.*, 2003a; Rice *et al.*, 1998; Sheldon *et al.*, 1997; Trommsdorff *et al.*, 1999).

In addition to the N terminal and central domain, the C terminal region of Dab1 also appears to be important for Reelin signaling. A mutant mouse expressing a truncated p45 Dab1 protein lacking this terminal domain displays a hypomorphic phenotype in heterozygosity (Herrick and Cooper, 2002). Unlike full-length p80 Dab1, which causes no discernable phenotype in heterozygous mice, a single gene copy encoding p45 Dab1 results in specific lamination defects. Heterozygous p45 Dab1 mice display a split in the hippocampal pyramidal layer and a hypercellular marginal zone in the neocortex, but no cerebellar defects. These observations suggest that the C terminus of Dab1 affects the strength of the Reelin signaling, possibly by promoting interaction with downstream effectors, in a cell type-specific manner.

C. Downstream of Dab1

Because disruption of SFK-dependent tyrosine sites on Dab1 results in a *reeler* phenotype (Howell *et al.*, 2000), it is generally believed that Dab1 phosphorylation is an event crucial for Reelin signal transduction, and not simply associated with degradation and termination of signaling. Therefore, a considerable effort has been dedicated to the identification of phospho-Dab1 interacting proteins that may transduce the downstream signal (see Fig. 5). A flurry of biochemical studies in the past few years demonstrated that phospho-Dab1 interacts with a variety of signaling proteins, all potentially important for modulating cytoskeletal dynamics, and thereby induces changes in neuronal migration and cellular

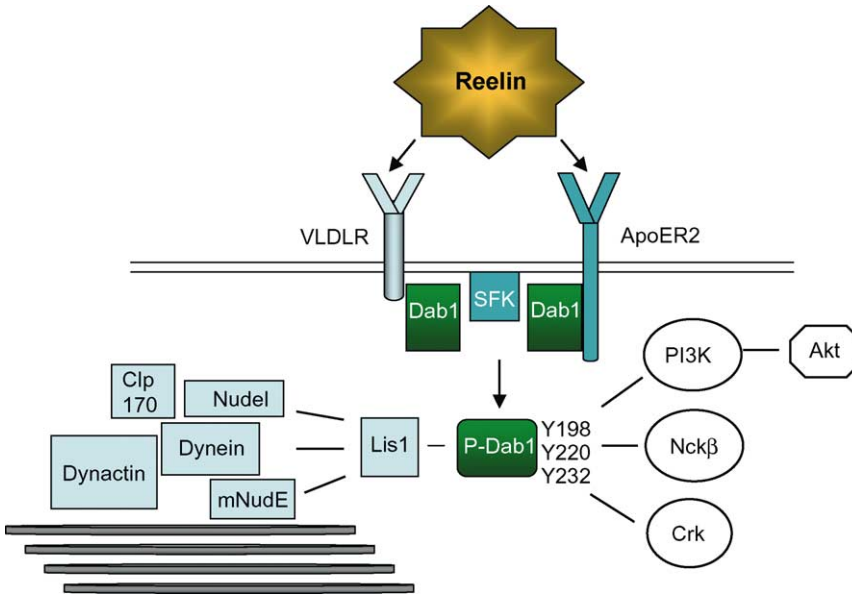

FIG. 5. The Reelin signaling pathway is activated by ligand-induced clustering of the Reelin receptors VLDLR and ApoER2, leading to SFK activation and Dab1 phosphorylation on the indicated tyrosine residues. Phospho-Dab1 interacts with a variety of intracellular proteins including Lis1, which ties the Reelin signal with the dynein complex and microtubule dynamics. Long bars indicate microtubules.

adhesion. Reelin was first found to activate the phosphatidylinositol-3-kinase (PI3K) and Akt, while inhibiting the glycogen synthase kinase (GSK) 3β, in an ApoER2/VLDLR- and Dab1-dependent manner (Beffert *et al.*, 2002). PI3K activation results from a direct interaction between phospho-Dab1 and the PI3K subunit p85α (Bock *et al.*, 2003). Pharmacological inhibition studies suggest that PI3K is required for Reelin-induced Akt activation and cortical plate formation *in vitro*, however the specific role of this kinase in Reelin signaling cannot be fully dissected from other signaling events linked to a multiplicity of cellular processes (Bock *et al.*, 2003). Phospho-Dab1 was also shown to bind Lis1, a protein involved in neuronal migration that is mutated in human Miller-Dieker lissencephaly (Hattori *et al.*, 1994; Reiner *et al.*, 1993). Lis1 participates in two major complexes. One enzymatic complex is the platelet-activating factor acetylhydrolase 1b (Pafah1b), in which Lis1 functions as a non-catalytic subunit. The other includes the microtubule motor dynein and dynein-associated proteins NudE, NudeL, dynactin, and CLIP-170 (reviewed by Vallee *et al.*, 2001; Wynshaw-Boris and Gambello, 2001). These proteins form an evolutionarily

conserved pathway that regulates centrosome function and mediates nucleokinesis. The interaction between phospho-Dab1 and Lis1 allows a cross-talk between Reelin signaling and Lis1-dependent molecular events that may be important for nuclear translocation and neuronal migration (Assadi *et al.*, 2003). The adapter molecule Nckβ can also bind phospho-Dab1 and redistribute to the membrane upon Reelin stimulation (Pramatarova *et al.*, 2003). This interaction is thought to cause remodeling of the actin cytoskeleton. Another link to the actin cytoskeleton is provided by the Dab1-binding protein N-WASP (Suetsugu *et al.*, 2004). This interaction results in the polymerization of actin and filopodia extension in non-neuronal cells. Interestingly, Dab1 phosphorylation was shown to suppress filopodia formation in this study, suggesting that a similar function in neurons may be important Reelin-induced termination of migration. A systematic screen of phospho-Dab1 binding proteins from embryonic brain lysates revealed interaction with PLC-γ1, Shp2, Src, CrkL, and CrkII (Ballif *et al.*, 2004). CrkL binds the Rap1 guanidine exchange factor C3G, which is phosphoryled on tyrosine residues in response to Reelin and leads to Rap1 activation. A similar screen of phospho-Dab1-binding protein resulted in the identification of RasGap, Nck-1, Nck-2, β-actin, CrkL, CrkII, and its splicing variant Crk-I (Huang *et al.*, 2004). Crk-I and CrkII, which are both products of the Crk gene, were shown to stimulate Dab1 phosphorylation in transfected cells in a Src-dependent way. Together, these studies suggest that Crk-family proteins form complexes with phospho-Dab1 and participate in Reelin signaling, both upstream and downstream of Dab1.

Other Dab1-intaracting proteins that do not require its phosphorylation include the amyloid precursor protein (APP) and APP-like proteins (Homayouni *et al.*, 1999; Howell *et al.*, 1999b), and the GTPase activating protein Dab2IP (Homayouni *et al.*, 2003). The significance of these interactions in Reelin signaling is unknown. In the case of APP, the link with Dab1 may be relevant to neurodegeneration. Mutations in genes encoding APP or APP-processing enzymes such as presenilin-1 and presenilin-2 cause increased phosphorylation of the axonal microtubule stabilizing protein tau and early onset Alzheimer's disease (Lee *et al.*, 2001). Interestingly, it has been shown that deficiency in Reelin, ApoER2/VLDLR, or Dab1 results in tau hyperphosphorylation and that APP is a genetic modifier of tau hyperphosphorylation in Dab1 mutant mice (Brich *et al.*, 2003; Hiesberger *et al.*, 1999). These findings reinforce the association between Reelin signaling and neurodegeneration previously established by the identification of ApoER2 and VLDLR as Reelin receptors as well as ApoE-binding proteins; ApoE proteins compete with Reelin for binding to these receptors, and the ApoE4 allele is a risk factor for late onset Alzheimer's disease. However, a specific effect of ApoE4 compared to ApoE2 and ApoE3 on Reelin binding could not be demonstrated using recombinant proteins (D'Arcangelo *et al.*, 1999).

Dab1 is generally thought to function downstream of Reelin receptors and to mediate signal transduction by virtue of its tyrosine phosphorylation-dependent association with many of the proteins previously mentioned. However, a recent study has raised the possibility that Dab1 may also function upstream of the receptors by regulating their trafficking to the plasma membrane (Morimura *et al.*, 2005). Cell-surface localization of Reelin was reduced in Dab1 mutant neurons, whereas cell surface expression of ApoER2 and VLDLR was augmented in Dab1-transfected cells. Phosphorylation of Dab1 appears to induce endocytosis of Reelin as well as its receptors and Dab1 itself, thus triggering a short-lived signaling cascade that terminates with Dab1 ubiquitination and degradation.

V. The Biological Activities of Reelin

A. Cellular Layer Formation

Despite advances in understanding the molecular steps involved in Reelin signaling, it is still not exactly clear how these events result in the formation of cortical layers in the developing brain. The presence of Reelin receptors and Dab1 in neuronal populations that become ectopic in *reeler* and *reeler*-like mutants suggests that the Reelin signal is passed on directly from the extracellular environment of superficial layers where Reelin is present to migrating neurons that approach these layers. The first contact is likely to be made on the leading edge of migrating neurons. However, the presence of Reelin signaling molecules on these cellular processes has not been demonstrated. This is due to technical difficulties related to the rapid trafficking of lipoprotein receptors at the plasma membrane, and the fact that the currently available antibodies against lipoprotein receptors and Dab1 work poorly in immunohistochemical assays. An alternative hypothesis is that radial fibers are the primary targets of Reelin and that neurons become disorganized as a secondary disruption of the radial scaffold. This view is based primarily on the observation that the radial scaffold is disrupted in *reeler* and *reeler*-like mutants. This disruption is particularly prominent in the dentate gyrus (Weiss *et al.*, 2003), but is more subtle in the neocortex where radial fibers appear to be shorter than normal and less branched (Derer, 1979; Hartfuss *et al.*, 2003). Addition of recombinant Reelin to the culture medium of hippocampal slices promoted radial fiber elongation (Zhao *et al.*, 2004). Interestingly though, it did not rescue layer formation and orientation of dentate granule cells. Granule cell migration could only be rescued by the addition of Reelin in a normotopic position near the marginal zone, indicating that Reelin is a positional cue that dictates proper positioning of dentate granule neurons, possibly by

acting primarily on radial fibers. In support of this hypothesis, several investigators have reported the expression of Reelin receptors and Dab1 in the ventricular zone of the neocortex where radial progenitor cells are abundant (Luque *et al.*, 2003; Rice *et al.*, 1998; Trommsdorff *et al.*, 1999) and in isolated progenitor cells (Hartfuss *et al.*, 2003). It should be noted, however, that the expression levels of Reelin transducing proteins in radial progenitor cells pail in comparison to those achieved in migrating neurons, particularly in superficial layers of the neocortex. Furthermore, in the cerebellum, ApoER2, VLDLR, and Dab1 expression has been described in Purkinje cells and not in radial cells, suggesting that Reelin acts directly on radially-migrating neurons in this structure. Nevertheless, it is possible that a defect in the radial scaffold, particularly in the dentate gyrus, contributes to layer disruption in the *reeler* brain.

To understand the molecular events that mediate Reelin function in cellular layer formation, several *in vitro* model systems have been developed. A classical model consists of cortical cell aggregates formed in rotating cultures. Cortical aggregates from *reeler* mice appear more disorganized than normal (DeLong, 1970; DeLong and Sidman, 1970), and normal aggregates can be rendered abnormal by the addition of the CR-50 interfering antibody (Ogawa *et al.*, 1995). Another assay system consists of isolated neurons lifted from embryonic cortical slices together with a radial fiber and cultured on a membrane (Dulabon *et al.*, 2000). Using this system it was reported that recombinant Reelin stops migration by causing detachment of neurons from the radial process. However, this conclusion was challenged based on the analysis of a transgenic mouse in which *reelin* is ectopically expressed in the ventricular zone from the nestin promoter (Magdaleno *et al.*, 2002). Expression of the transgene in the ventricular zone did not cause an arrest of neuronal migration in normal mice, and partially rescued the cortical phenotype in *reeler* mice. These findings suggest that Reelin acts more as an instructive molecule, enabling neurons to respond to positional cues, than a physical barrier. However, it is also possible that progenitor cells in the ventricular zone of transgenic mice transported the Reelin protein along the radial fiber and secreted it in the marginal zone, the normotopic region of the cortex, where it could function as a positional cue by signaling the end of migration. Expression studies in several regions of the central nervous system are consistent with the view that Reelin represents a stop signal to radial migration. Also supporting this is the fact that the Reelin protein is seen in marginal areas of the forebrain, cerebellum, and spinal cord where Dab1-positive neurons terminate (Kubasak *et al.*, 2004; Rice *et al.*, 1998; Yip *et al.*, 2004a).

A recently developed assay for neocortical development involves embryonic cortical slices in which two consecutive neuronal cohorts are labeled with radioactive thymidine and BrdU (Jossin *et al.*, 2003b). This model enabled investigators to examine early events in corticogenesis, such as preplate splitting and the establishment of an inside-out gradient. Addition of recombinant full-length

Reelin to the medium rescued the *reeler* defects. Fragments of recombinant Reelin similar to those generated spontaneously by proteolytic processing could be analyzed for the first time for their biological activity. This analysis led to the identification of a central domain of Reelin (consisting of repeats 3 to 6) that is necessary and sufficient for preplate splitting and early layer formation (Jossin *et al.*, 2004). N terminal fragments (up to repeat 3) or C terminal fragments (repeats 6 to 8) were not functionally active. These functional data correlated well with the ability of Reelin fragments to induce Dab1 phosphorylation in cortical slices. The central domain, even though sufficient for activity, appears to be less effective than the full-length Reelin protein. One possible explanation for this difference is that the CR50 epitope, which is absent in the central fragment but mediates the aggregation of full-length Reelin in multimers, may potentiate the biological activity of Reelin.

The cortical slice system was also exploited in a pharmacological approach to demonstrate the requirement for SFKs, non-classical protein kinase C (PKC) and PI3K in layer formation (Bock *et al.*, 2003; Jossin *et al.*, 2003b). Addition of the SFK inhibitor PP2 and the protein kinase C inhibitor bisindolylmaleimide 1 (BIM1) to wild type cultures prevented normal corticogenesis. PP2 also prevented Dab1 phosphorylation in cortical slices, as previously demonstrated in dissociated neurons (Arnaud *et al.*, 2003b; Bock and Herz, 2003), whereas BIM1 did not (Jossin *et al.*, 2003b). The role of non-classical PKCs in Reelin signal transduction has not been further elucidated. Addition of the PI3K inhibitor LY294002 also blocked the formation of a well-defined cortical plate and Akt phosphorylation (Bock *et al.*, 2003), indicating that activation of this pathway by Reelin or clustered receptors (Ballif *et al.*, 2003; Beffert *et al.*, 2002; Strasser *et al.*, 2004), is physiologically relevant to corticogenesis.

To visualize live migrating neurons in cortical slices, techniques were developed to label neurons with fluorescent dyes *in vitro* (Nadarajah *et al.*, 2001) or *in vivo* by *in utero* electroporation (Tabata and Nakajima, 2001). The migration of selected labeled neurons was then conducted by time-lapse confocal imaging. Using this approach, it was discovered that neurons migrate by three modalities, radial fiber-guided locomotion, somal translocation, and branched migration (Nadarajah *et al.*, 2001; Tabata and Nakajima, 2003). The two latter modes of migration, which had not previously been described, enable neurons to move without the support of the radial fiber scaffold. Somal translocation appears to be commonly used by migrating neurons in the beginning of corticogenesis: a long leading edge extends deep into the preplate followed by its collapse, which brings the cell body in a superficial position (Nadarajah *et al.*, 2001).

One limitation of the cortical slice system is that it is a short-term assay, allowing analysis of corticogenesis only for 3 days *in vitro*. To examine later stages of corticogenesis, *in utero* gene transfer of GFP-expressing vectors was used to label cohorts of newborn neurons in mouse embryos that were carried to term.

When GFP-labeled neurons were visualized in the postnatal *reeler* cortex, they appeared to be unable to reach the top of the cortex and developed abnormally oriented dendrites in abnormally deep positions (Tabata and Nakajima, 2002). Later born neurons tended to accumulate along dendrite-rich areas, which in normal cortex consist largely of the marginal zone. However, in *reeler,* ectopic dendrites clustered throughout the cortex and appeared to impede the progress of migrating neurons toward the surface of the cortex. These observations suggest that defective dendrite growth may also contribute to cortical layer disruption in the *reeler* cortex. A further improvement of this assay system was achieved by combining *in utero* gene transfer with time-lapse confocal imaging of cortical slices. The use of GFP-carrying retroviruses injected *in utero,* allowing for clonal analysis of dividing cells and their progeny, was instrumental in demonstrating that radial cells are precursors for both neurons and glia at different stages of corticogenesis (Malatesta *et al.*, 2000; Noctor *et al.*, 2001). When this approach was applied to Dab1-deficient *scrambler* mice (Sanada *et al.*, 2004), mutant neurons were found to be unable to extend their leading edge into the preplate. This observation suggests that Reelin may have an attractive or stabilizing function on these cellular processes, likely mediated by phospho-Dab1 and its interacting proteins through modifications of the cytoskeleton. Mutant *scrambler* neurons also appeared to be abnormally closely associated to radial fibers. Proper positioning of GFP-labeled neurons was shown to be dependent on intrinsic Dab1 expression in migrating neurons and was not inhibited by the presence of Dab1-deficient radial fibers in the *scrambler* cortical background. This provides evidence that Reelin functions by signaling to migrating neurons directly, and not indirectly through radial fibers, at least in the neocortex. Since the mutant environment did not interfere dramatically with the radial progression of Dab1-expressing neurons, this study demonstrates that obstruction due to increased adhesion to radial fibers cannot alone account for the cortical layer inversion seen in *reeler* or Dab1 mutant cortex. Dab1 phosphorylation on tyrosine residues 220 and 232 was further shown to be essential for proper detachment from radial fibers, as expression of these Dab1 proteins mutated on these sites did not rescue the *scrambler* defect (Sanada *et al.*, 2004). These mutant Dab1 proteins had a dominant negative effect, as they caused excessive adhesion to radial fibers even in the presence of normal Dab1 in the wild type cortex. One possible mechanism for increased adhesion appears to be the upregulation of $\alpha 3$ integrin. In the *scrambler* cortex $\alpha 3$ integrin levels were increased (Sanada *et al.*, 2004). It is not yet known whether a similar upregulation is also present in the *reeler* cortex. The excessive adhesion of tyrosine 220 mutant Dab1-expressing neurons could be countered by downregulation of $\alpha 3$ integrin levels with RNA interference, indicating that the detachment of migrating neurons from radial fibers is controlled by this integrin. It is interesting to note that $\alpha 3$ integrin in association with the $\beta 1$ chain has been shown to bind Reelin and Dab1 (Dulabon *et al.*, 2000;

Schmid *et al.*, 2005). However, adhesion control does not appear to be a key requirement for cortical layer formation, since lamination proceeds fairly normally in $\alpha 3$ (Anton *et al.*, 1999) and $\beta 1$ (Graus-Porta *et al.*, 2001) knock out mice.

In summary, current data indicate that Reelin promotes cortical layer formation by a combination of distinct molecular mechanisms. By acting primarily on migrating neurons, the Reelin signal mediated by VLDLR/ApoER2 and Dab1 may promote the extension and stabilization of the leading edge into the marginal zone, which results in migration arrest and proper orientation. Reelin signal also may induce a decrease in neuronal adhesion to radial fibers through $\alpha 3\beta 1$ integrin downregulation, facilitating the assembly of cellular layers. An additional effect of Reelin on the stability of the radial scaffold may also contribute to proper neuronal migration and cortical lamination.

B. Neuronal Maturation

In addition to its function as a key regulator of neuronal positioning, Reelin also functions as a neurotrophic factor that promotes growth and maturation of cellular processes. Earlier studies demonstrated that the axonal projections of the entorhinohippocampal pathway are defective in *reeler* mice, and that addition of the CR-50 antibody interferes with their development in normal hippocampal slices (Del Rio *et al.*, 1997). The extent of terminal branching appeared to be transiently affected in *reeler*. So far, this observation has not been extended to other fiber projection systems. The lamina-specific termination of the hippocampal projections is known to be strictly dependent on the position of the target cells, which is aberrant in *reeler* (Deller *et al.*, 1999). Thus, it appears that Reelin only indirectly affects axonal projections. Consistent with this view, it was observed that axonal targeting and fasciculation of vomeronasal projections into the accessory olfactory system are normal in *reeler* (Teillon *et al.*, 2003). Furthermore, no effect on axon outgrowth was observed using cortical explants cultured in a collagen gel, a system that enables to readily detect axon repulsion by semaphorins (Jossin and Goffinet, 2001).

Unlike axons, dendritic processes are severely affected in *reeler* brain. Earlier Golgi impregnation studies revealed the stunted and disoriented dendritic trees of cortical, hippocampal, and cerebellar neurons (Mikoshiba *et al.*, 1980; Pinto Lord and Caviness, 1979; Stanfield and Cowan, 1979). Other studies using *in utero* electroporation of GFP-expression plasmids showed that dendrite growth is delayed in the *reeler* neocortex (Tabata and Nakajima, 2002). Recent studies further demonstrated that dendrite defects in *reeler* are not only secondary to cellular ectopia, but are, at least in part directly due to the absence of trophic activity of Reelin. Dissociated hippocampal cultures from *reeler* mice, as well as from Dab1 mutant mice, exhibited reduced dendrite development (Niu *et al.*,

2004). The deficit was observed not only in homozygous mutant mice, but also in developing heterozygous mice that exhibit no positional defects. The *reeler* defect could be rescued by the addition of Reelin, whereas lipoprotein receptor antagonists and SFK inhibitors blocked normal growth (Niu *et al.*, 2004). These data indicated that the same signaling pathway that controls neuronal positioning during embryogenesis also controls dendrite growth in the postnatal brain.

Neuronal maturation during postnatal development involves not only the extension of cellular processes but also the formation of synapses. As previously mentioned, synaptic circuitry is disrupted in the *reeler* retina in the absence of cellular layer defects (Rice *et al.*, 2001). Interestingly, the density of dendritic spines appears reduced in the prefrontal cortex of heterozygous *reeler* mice, in the absence of cellular ectopia (Liu *et al.*, 2001). It is not currently known whether this synaptic deficit is widespread, nor whether it is secondary to delayed dendrite development. Reelin has been shown to induce LTP in hippocampal slice cultures, further suggesting a role in regulating synaptic function in the postnatal and adult brain (Weeber *et al.*, 2002). This Reelin-induced form of plasticity was shown to be dependent on ApoER2 and VLDLR, indicating that the Reelin signaling pathway remains active throughout brain development, even though its biological function changes from the control of neuronal migration and cellular architecture in the prenatal brain to the regulation of neuronal maturation and synaptic activity in the postnatal brain.

VI. Reelin and Human Diseases

The *reelin* gene is highly conserved among vertebrate species, including humans (DeSilva *et al.*, 1997). In the primates and human brain, *reelin* expression pattern is very similar to that of rodents (Meyer and Goffinet, 1998; Rodriguez *et al.*, 2000). Thus, it is very likely that in humans Reelin plays roles similar to those proposed above based on animal models. Homozygous loss of Reelin in humans causes a severe neurological disease known as lissencephaly with cerebellar hypoplasia (LCH) (Hong *et al.*, 2000). The phenotype of these patients is reminiscent of that of *reeler* mice, and is characterized by profound ataxia. In addition, LCH patients also display seizures, severe cognitive deficits, and lymphoedema. The reduction in the number of cortical gyri (lissencephaly) is a uniquely human feature, due to the absence of gyration in the mouse cortex. The similarity between lissencephaly due to Reelin absence and that due to Lis1 haploinsufficiency is likely explained by the cross-talk between signaling pathways controlled by these molecules (Assadi *et al.*, 2003).

Alterations of *reelin* expression not due to inherited genetic mutations have also been reported in developmental brain abnormalities affecting the cortex and

the hippocampus. An excessive number of Reelin-expressing cortical Cajal-Retzius cells were observed in abnormal brain regions of patients with some forms of cortical dysplasia and polymicrogyria, conditions associated with epilepsy (Eriksson *et al.*, 2001; Garbelli *et al.*, 2001). On the other hand, decreased expression of *reelin* by hippocampal Cajal-Retzius cells correlated with granule cell dispersion in the dentate gyrus of patients with temporal lobe epilepsy (Haas *et al.*, 2002). Together, these findings suggest that altered *reelin* expression may result in neuronal migration defects associated with different forms of epilepsy.

A decrease in *reelin* mRNA expression is also consistently seen in patients with schizophrenia and bipolar disorder, leading to the suggestion that Reelin deficiency may be a risk factor for these diseases (Guidotti *et al.*, 2000; Impagnatiello *et al.*, 1998). Low levels and altered distribution of Reelin-expressing cells was also observed in the white matter of schizophrenic tissue (Eastwood and Harrison, 2003), although no differences in Reelin expression in the serum of patients with neurological disease compared to control subjects were detected (Ignatova *et al.*, 2004). No detailed information is currently available on the health status of heterozygous carriers of *reelin* mutations, however they do appear to display overt neurological dysfunctions or mental illness. Because heterozygous *reeler* mice, like patients with schizophrenia and bipolar disorders, express reduced levels of Reelin (D'Arcangelo *et al.*, 1995), they have been proposed to be good models for the diseases (Costa *et al.*, 2002). Some behavioral studies of heterozygous *reeler* mice reported deficits using tests that specifically address anxiety and fear, such as prepulse inhibition of startle, and neophobic behavior, which resemble traits of schizophrenia (Tueting *et al.*, 1999). Alterations in the mesotelencephalic dopamine pathway have also been reported in heterozygous *reeler*, which may be relevant to psychotic disorders (Ballmaier *et al.*, 2002). However, other investigators have reported no behavioral deficits in heterozygous *reeler* mice using a battery of tests for evaluating emotional traits and memory (Podhorna and Didriksen, 2004; Salinger *et al.*, 2003). Genetic studies so far have also failed to demonstrate linkage between the *reelin* locus on chromosome 7q22 and schizophrenia or bipolar disorders. This could be explained by the observation that *reelin* expression is controlled by epigenetic factors such as promoter methylation (Chen *et al.*, 2002; Tremolizzo *et al.*, 2002). DNA methyltransferase (Dnmt) 1 was shown to regulate *reelin* gene expression (Noh *et al.*, 2005). Interestingly, the expression of this gene appears to be increased in cortical GABAergic interneurons of schizophrenic brains (Veldic *et al.*, 2004). This upregulation correlates with the decrease in *reelin* mRNA expression in the same interneurons. These findings suggest that promoter hypermethylation of *reelin*, and perhaps of other genes expressed in these interneurons, is associated with schizophrenia. The recent observation that histone deacetylase inhibitors decrease *reelin* promoter methylation raises the possibility that these drugs might be used in the treatment of psychoses (Mitchell *et al.*, 2005; Tremolizzo *et al.*, 2005).

Other studies suggested a link between *reelin* polymorphisms and autism (Persico *et al.*, 2001; Skaar *et al.*, 2004; Zhang *et al.*, 2002). This link is currently being further investigated (see this volume). If a cause-and-effect relationship between *reelin* and psychiatric diseases is established, a plausible mechanism may be synaptic dysfunction due to a postnatal Reelin deficit in the developing brain of these patients, rather than defective neuronal migration in early embryogenesis.

In conclusion, studies of the *reeler* mutation have shed some light on many diverse aspects of brain development. These mutant mice will continue to serve as invaluable models for neuronal migration, maturation, and synaptic circuitry, and the Reelin signaling pathway will continue to serve as a springboard for the investigation of the molecular events underlying these processes. Given the association between Reelin and a variety of human developmental brain disorders, these studies also hold great promise for the development of new treatments for these devastating conditions.

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SHARED CHROMOSOMAL SUSCEPTIBILITY REGIONS BETWEEN AUTISM AND OTHER MENTAL DISORDERS

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We have compiled significant results from 53 genome scans for five different mental disorders including autism syndrome disorder (ASD), schizophrenia (SZ) and catatonia, bipolar disorder (BP), attention-deficit/hyperactivity disorder (ADHD), and alcoholism. Eight autosomal chromosomes (1, 2, 3, 7, 9, 13, 15, and 17) showed significant linkages with ASD while five of these chromosomes (1, 7, 13, 15, and 17) shared common susceptibility loci with other mental disorders mostly SZ and BP. Chromosome 15 is particularly rich in shared regions, three to four being detected where all four other mental disorders are involved in one or the other of these regions. Chromosome 15 is a particularly unstable chromosome where numerous chromosomal rearrangement and abnormalities have been associated with ASD. Strong candidate genes such as gamma-amino-butyric acid (GABA) receptor B3, A5, and G3 have shown associations with ASD on this chromosome. Some susceptibility loci for different mental disorders have also been assigned to chromosome 15 such as schizophrenia 10, major depressive disorder 2, dyslexia 1, epilepsy juvenile myotonic 2, and spinocerebellar ataxia 11. Finally, the only significant linkage results with catatonia are also found in a region shared by ASD, SZ, and ADHD on chromosome 15.

I. Introduction

Autism spectrum disorder (ASD) is a severe neurodevelopmental disorder characterized by delayed or absent speech, impairments in social interaction and in communication, and repetitive behaviors and restricted interests. The relationships between ASD, schizophrenia (SZ), and bipolar disorder (BP) are not clear.

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Although characteristic features of SZ and BP such as delusions, hallucinations, euphoria, or melancholia are usually not present in people with ASD, there are also a few common psychopathological dimensions. For example, catatonia has been described in ASD, SZ, and BP (Abrams and Taylor, 1976; Dhossche, 1998; Realmuto and August, 1991). Moreover, family studies have reported increased rates of schizophrenia-like and affective disorders (Larsson *et al.*, 2005).

Parental schizophrenia-like psychosis and affective disorder were significant risk factors for autism in offspring in a nationwide Danish case-control study of 698 children diagnosed with autism between 1972–1999 (Larsson *et al.*, 2005). Relative risks were 3.44, 95% CI 1.48–7.95 and 2.91, 95% CI 1.65–5.14, for parental schizophrenia-like disorder and affective disorder respectively. Other significant variables were breech presentation (RR = 1.63), low Apgar score at 5 minutes (RR = 1.89), and gestational age at birth less than 35 weeks (RR = 2.45). Weight for gestational age, parity, number of antenatal visits, parental age, or socioeconomic status were not significant risk factors. These findings support that perinatal factors and parental psychopathology are associated with risk of autism. It remains an open question whether perinatal adversity was due to environmental factors, factors associated with autism in the fetus, or a combination of these and possibly other (unmeasured) variables.

A parsimonious explanation is that the genetic make-up of the fetus interferes with intrauterine development leading to increased risk for perinatal complications. However, perinatal factors and parental psychiatric disorder seemed to act independently in this study. This suggests two sets of autism-related etiologies (i.e., a genetic set and an obstetric set). These findings need to be replicated in other samples. However, the Danish study will be hard to match because of its almost complete ascertainment of cases, high quality of information on all risk factors, and prospective design. In any case, further family psychiatric studies assessing psychotic disorders, including catatonic subtypes, as risk factors for autism are warranted. Previous studies have typically not separated out catatonic subtypes of schizophrenia, affective disorder, or other psychotic disorders.

A subtype of unsystematic SZ is characterized by periodic catatonia where acute psychotic episodes are followed by remission. These disorders have shown strong heritabilities with some 15 to 20 genes expected to be involved in ASD (Spence, 2004), while a major gene effect is predicted for catatonia (Stober *et al.*, 1995). The search of the genes related to these psychiatric disorders has shown significant progress in the recent years with the identification of some strong candidates for SZ such as the dystrobevin binding protein 1 (Straub *et al.*, 2002) and the neuregulin 1 (Stefansson *et al.*, 2002) genes. Numerous other genes have been tested for these disorders with more or less success. However, from the literature, it is obvious that the same genes and biological pathways could be shared among the disorders. A striking example is the catechol-O-methyltransferase gene (COMT) on chromosome 22q11.2. COMT encodes a key enzyme in

the elimination of dopamine in the prefrontal cortex of the human brain and this role in the degradation of catecholamine neurotransmitters may suggest a general involvement of COMT in psychiatric diseases. The COMT protein shows two forms, the membrane-bound longer form being the main form expressed in the brain, while the soluble shorter form is expressed in other tissues such as the spleen and the liver. A common COMT polymorphism (Val108/158Met) in exon 4, changing a valine for a methionine at the position 108 or 158 of the short and the long forms, respectively, affects significantly the protein abundance and the enzyme activity, but not mRNA expression (Chen *et al.*, 2004a; Egan *et al.*, 2001; Shield *et al.*, 2004). Positive association of Val108/158Met with SZ have been generally reported (Chen *et al.*, 2004b; Egan *et al.*, 2001; Glatt *et al.*, 2003a; Sazci *et al.*, 2004; Wonodi *et al.*, 2003), as weaker evidence in a recent meta-analysis (Fan *et al.*, 2005), and in a study of Korean SZ inpatients (Park *et al.*, 2002). COMT Val108/158Met polymorphism has also been associated with schizotypy (Avramopoulos *et al.*, 2002) but not cognition (Stefanis *et al.*, 2004), with prefrontal neurocognitive function in healthy (Rosa *et al.*, 2004) but not SZ individuals (Ho *et al.*, 2005; Rosa *et al.*, 2004), with the 22q11.2 deletion syndrome (Bearden *et al.*, 2004), with anxiety (Enoch *et al.*, 2003; McGrath *et al.*, 2004), and with anorexia nervosa (Gabrovsek *et al.*, 2004). Two SNPs located in intron 1 and in the 3′ flanking region of COMT have also been associated with a risk for SZ (Shifman *et al.*, 2002) and for BP (Shifman *et al.*, 2004). It has been proposed that the effect of the intron 1 SNP in SZ could come from a linkage disequilibrium (LD) with a SNP in the P2 promoter (Palmatier *et al.*, 2004). These SNPs lowered COMT mRNA expression in anonymous postmortem brains (Bray *et al.*, 2003), while this lower expression was not observed in the lymphocytes of SZ patients (Chen *et al.*, 2004a). Other examples of the communality observed between mental disorders are the serotonergic and dopaminergic family of genes. For example, the serotonin transporter gene has been associated with different mental disorders including ASD (Yirmiya *et al.*, 2001), while the dopamine receptor D2 had showed association with attention-deficit/hyperactivity disorder (ADHD), alcoholism (ALC), and Tourette's syndrome (Comings *et al.*, 1991).

We have compiled the significant results from published genome scans for six different mental disorders and observed shared chromosomal susceptibility regions among all of them (Chagnon *et al.* in preparation). From this first analysis, we observed that chromosome 15, particularly, shared common susceptibility regions for some disorders including ASD, SZ, BP, ADHD, and ALC. The only significant linkage results for catatonia in a subgroup of SZ were also observed on chromosome 15. In this chapter, we will present chromosomes with significant genome scan results for ASD putting an emphasis on chromosome 15 where chromosomal rearrangements and abnormalities, and candidate gene analyses will also be presented in relation to ASD and catatonia susceptibility loci.

A. Methods

Relevant genome scan papers have been identified by a search in the PubMed database using the key words "linkage OR genome scan" AND "schizophrenia OR bipolar disorder OR autism OR catatonia OR alcoholism OR attention deficit hyperactivity deficiency OR Tourette" (Chagnon *et al.* in preparation). The criterion for inclusion of the results of a genome scan was a Lod score of 3.0 and greater corresponding to a P value of 0.0001 and smaller. P values of 0.05 and smaller were also included when "genome wide adjusted." From these, only chromosomes including significant susceptibility regions for ASD have been retained in the actual compilation. The chromosomal location of all the linked markers has been updated using the same and most recent version of the physical map in megabases (Mb) from the National Center for Biological Information (NCBI built 35.1). One Mb corresponds to 10^6 bases or nucleotides of DNA. When the location on the physical map was not available for a given marker, genetic maps in centimorgan (cM) units from Marshfield (Broman *et al.*, 1998) have been used to determine the relative position of the marker, where one cM corresponds roughly to 1 Mb. Additionally, the cytological locations have been updated using the predictive locations (GMAP) from the Genetic Location Database (Collins *et al.*, 1996). We have also reported for the linked markers of these chromosomes the susceptibility loci assigned by NCBI (built 35.1) where the same locus can be assigned to more then one marker. For example, four linked markers of the genome scans (D1S1631, D1S1653, D1S1679, and D1S196) are related to the Schizophrenia susceptibility 9 on chromosome 1p21.2-q24.2, while a unique linked marker (D1S484) at 1q24.1 is related to the Asperger syndrome 3. Some apparent contradictions are observed between physical and cytological locations for these susceptibility loci. For example, Autism 1 susceptibility locus at 7q31.31 is related to marker D7S486 according to NCBI ePCR result while Autism 1 was originally assigned to 15q11-q13. For chromosome 15 only, ASD and catatonia suggestive genome scan results, chromosomal rearrangements and abnormalities, and positive or negative candidate gene analyses have also been included.

B. Results and Discussion

1. *Shared Chromosomes*

Table I presents the results from the 53 genome scans related to ASD (N = 18), to SZ (N = 17), to SZ catatonia (N = 2), to SZ and BP (N = 4), to BP (N = 8), to ADHD (n = 1), and to ALC (N = 3) that have been included in the table. We observed that eight autosomal chromosomes (1, 2, 3, 7, 9, 13, 15, and 17) showed significant linkages with ASD (see Table I). Chromosome 15

TABLE I

SIGNIFICANT GENOME SCAN RESULTS OBSERVED FOR ASD AND SHARED BY OTHER MENTAL DISORDERS (MD). PHYSICAL (NCBI) AND GENETIC (MARSHFIELD) ARE REPORTED

Chr	Location	Markers	NCBI	Marshfield	Statistic	MD	Loci/ Association results	References
1	1p36.32	D1S1612	8052251	16.2	Lod = 3.3	SZ		(Abecasis *et al.*, 2004)
1	1p21.2	D1S1631	105372673	136.9	Zmax = 3.4	ASD	SCZD9	(Risch *et al.*, 1999)
1	1q21.3	KCNN3	151655827	na	ns	SZ catatonia		(Stober *et al.*, 2000a)
1	1q22	D1S1653	154745847	164.1	Lod = 6.1	SZ	SCZD9	(Brzustowicz *et al.*, 2000)
1	1q24.1	D1S484	157580640	169.7	Zmax = 3.6	ASD (AS)	ASPG3	(Ylisaukko-Oja *et al.*, 2004)
1	1q24	rs1415263	158897701	na	Lod = 6.5	SZ		(Brzustowicz *et al.*, 2002)
1	1q24	336H14-CA1	158897701	na	p = 0.001	SZ		(Brzustowicz *et al.*, 2004)
1	1q24.2	D1S1679	159093573	170.8	Lod = 6.1	SZ	SCZD9	(Brzustowicz *et al.*, 2000)
1	1q24.2	D1S196	164791505	181.5	Lod = 3.2	SZ	SCZD9	(Gurling *et al.*, 2001)
1	1q31.2	PFKFB2	203639264	na	p = 0.04*	SZ		(Stone *et al.*, 2004)
1	1q42.12	D1S2141	211583695	233.4	Zmax = 3.8	SZ		(Hovatta *et al.*, 1999)
1	1q42.2	D1S2709	228337174	247.2	Zmax = 3.2	SZ		(Ekelund *et al.*, 2001)
2	2p14	D2S441	68150649	na	Lod = 3.2	BP	PARK3	(Liu *et al.*, 2003)
2	2p11.1	D2S139	79675251	101.6	Lod = 3.0	SZ		(DeLisi *et al.*, 2002)
2	2p11.1	D2S1790	84986947	na	Lod = 4.2	SCI/ALC		(Hesselbrock *et al.*, 2004)
2	2q31.1	D2S335	172392096	175.9	NPL = 3.3	ASD		(Buxbaum *et al.*, 2001)
2	2q31.3	D2S2188	175430218	180.8	MLS = 4.8	ASD		(IMGSAC, 2001a)
2	2q32.1	D2S364	182860040	186.2	NPL = 3.3	ASD	AUTS5	(Buxbaum *et al.*, 2001)
2	2q37.3	D2S427	232031769	236.7	Zmax = 4.4	SZ		(Paunio *et al.*, 2001)
3	3p11.1	D3S1276	85338848	111.9	NPL = 3.6	BP		(Bailer *et al.*, 2002)
3	3q11.1	D3S1271	102217427	na	NPL = 3.2	BP		(Bailer *et al.*, 2002)
3	3q27.1	D3S3037	na	190.4	Lod = 4.3	ASD	AUTS3 ASPG1	(Auranen *et al.*, 2002)
3	3q29	D3S1265	197014377	222.8	NPL = 3.7–3.5	SZ/BP		(Bailer *et al.*, 2002)
3	3q29	D3S1265	197014377	222.8	NPL = 4.0	SZ/BP		(Schosser *et al.*, 2004)
3	3q29	D3S3550	na	227.6	NPL = 4.1–3.9	BP		(Bailer *et al.*, 2002)
7	7p11.2	D7S1818	49166136	69.6	MLS = 3.0	ADHD		(Bakker *et al.*, 2003)
7	7q31.1	D7S501	106034415	118.9	MLS = 3.5	SZ/BP		(Ekelund *et al.*, 2000)

(*Continued*)

TABLE I (*Continued*)

Chr	Location	Markers	NCBI	Marshfield	Statistic	MD	Loci/ Association results	References
7	7q31.31	D7S523	111295678	123.0	MLS = 3.5	SZ/BP		(Ekelund *et al.*, 2000)
7	7q31.31	D7S486	115488713	124.1	MLS = 3.2	SZ	AUTS1	(Ekelund *et al.*, 2000)
7	7q32.1	D7S530	128216773	134.6	MLS = 3.6	ASD	AUTS1	(IMGSAC, 1998)
7	7q	D7S684	137521669	147.2	MLS = 3.6	ASD	AUTS1	(IMGSAC, 1998)
7	7q34	D7S1824	139465749	149.9	Z = 3.0	ASD		(Alarcon *et al.*, 2002)
7	7q36.2	D7S2462	152999336	169.8	Lod = 3.7	ASD		(Auranen *et al.*, 2002)
9	9p24.2	D9S288	3941795	na	Lod = 3.1	SZ		(Wilcox *et al.*, 2002)
9	9p22.3	D9S157	17618382	32.2	MLS = 3.1	ASD		(IMGSAC, 2001a)
9	9q12	D9S301	71032274	na	p = 0.0005	ALC		(Bergen *et al.*, 2003)
9	9q34.3	D9S1826	135674269	159.6	MLS = 3.6	ASD		(IMGSAC, 2001a)
9	9q34.3	D9S158	136325009	161.7	MLS = 3.2	ASD		(IMGSAC, 2001a)
13	13q13.3	D13S1491	37461442	na	Lod = 3.0	SZ		(Maziade *et al.*, 2005)
13	13q14.2	D13S1272	43983647	41.7	NPL = 4.1	BP		(Badenhop *et al.*, 2001)
13	13q14.3	D13S153	47788774	45.6	NPL = 4.1	BP		(Badenhop *et al.*, 2001)
13	13q22.32	D13S800	72772693	55.3	MML = 3.0	ASD		(IMGSAC, 2001b)
13	13q31.1	D13S317	81620060	63.9	Lod = 3.6	PBP	SCZD7	(Potash *et al.*, 2003)
13	13q32.1	D13S793	na	na	Lod = 4.4	SZ	SCZD7; PAND	(Brzustowicz *et al.*, 1999)
13	13q32.3	D13S1271	na	79.5	Lod = 3.4	BP		(Detera-Wadleigh *et al.*, 1999)
13	13q32.3	D13S779	100301956	82.9	Lod = 3.4	BP	SCZD7	(Detera-Wadleigh *et al.*, 1999)
13	13q32.3	D13S174	101752077	84.9	NPL = 4.2	SZ	SCZD7	(Blouin *et al.*, 1998)
15	15q11.2	D15S128	22681893	6.1	Z = 4.0	SZ	SCZD10	(Freedman *et al.*, 2001)
15	15q11.2	D15S128	22681893	6.1	Lod = 0.7	ASD	SCZD10	(Philippe *et al.*, 1999)
15	15q11.1	D15S122	23231137	6.1	Lod = 4.6	BP	UBE3A	(Maziade *et al.*, 2005)
15	15q12	UBE3A	23231137	6.1	p = 0.004	ASD	LD, T_{SP} 5′ UTR D15S122	(Nurmi *et al.*, 2001)
15	15q12	UBE3A	23235221	na	ns	ASD	Angelman syndrome	(Veenstra-VanderWeele *et al.*, 1999)

15	15q	ATP10A[#]	23659963	na	p = 0.03, 0.03	ASD	PDT exon 22 rs1047700, intron 2 rs1345098	(Nurmi *et al.*, 2003)
15	15q11.1	GABRB3	24570020	na	Lod = 4.7	ASD		(Shao *et al.*, 2003)
15	15q11.1	GABRB3	24570020	na	p = 0.001	ASD	MTDT 155CA-2	(Cook *et al.*, 1998)
15	15q11.1	GABRB3	24570020	na	ns	ASD	TDT 155CA-2	(Maestrini *et al.*, 1999)
15	15q11.1	GABRB3	24570020	na	p = 0.01, 0.04 p = 0.03, 0.04	ASD	PDT intron 7 rs1432007, hCV2901140 PDT rs4542636, rs878960	McCauley *et al.*, 2004)
15	15q11.1	GABRA5	24724386	na	p = 0.03	ASD	TDT intron 6 hCV252720	(McCauley *et al.*, 2004)
15	15q12	GABRG3	24799413	na	p = 0.02, 0.03	ASD	exon5_539T/C, intron5_687T/C	(Menold *et al.*, 2001)
15	15q11.2	D15S217	25747918	na	Z = 1.8	ASD		(Bass *et al.*, 2000)
15	15	APBA2	27000145	na	ns	ASD		(Sutcliffe *et al.*, 2003)
15	15q13.3	D15S1360 (CHRNA7)	(30110018)	na	Lod = 5.3	SZ/P50		(Freedman *et al.*, 1997)
15	15q15.3	ACTC	32870697	31.5	Lod = 3.5	BP		(Turecki *et al.*, 2001)
15	15q15.3	D15S118	34024135	32.6	Z = 4.0	SZ	SCZD10; EJM2	(Freedman *et al.*, 2001)
15	15q15.3	D15S118	34024135	32.6	Lod = 1.1	ASD	SCZD10; EJM2	(Philippe *et al.*, 1999)
15	15q15.3	D15S1042	34050903	32.6	Z = 2.6, 3,9	SZ catatonia		(Stober *et al.*, 2000b, 2002)
15	15q15.3	GATA50C03	na	34.8	MLS = 3.5	ADHD		(Bakker *et al.*, 2003)
15	15q21.1	D15S1012	36794835	36.0	Z = 2.8	SZ catatonia		(Stober *et al.*, 2000b)
15	15q21.1	SLC30A4	43602294	na	na	SZ catatonia	No variant detected in coding and promotor sequences	(Kury *et al.*, 2003)
15	15q21.1	D15S659	44161300	43.5	Z = 3.9	SZ catatonia		(Stober *et al.*, 2002)
15	15q21.2	D15S117	56266889	51.2	MLS = 2.6	ASD	Non-male affected sib-pairs	(Lamb *et al.*, 2005)

(*Continued*)

TABLE I (*Continued*)

Chr	Location	Markers	NCBI	Marshfield	Statistic	MD	Loci/ Association results	References
15	15q22.31	D15S125	64880007	64.2	MLS = 2.6	ASD	Non-male affected sib-pairs	(Lamb *et al.*, 2005)
15	15q22.31	PKM2 (PK3)	70278439	na	p = 0.02*	SZ		(Stone *et al.*, 2004)
15	15q26.2	D15S652	90318371	90.0	Z = 2.3	ASD	MDD2	(Risch *et al.*, 1999)
15	15q26.3	D15S1014	95803594	107.7	Lod = 4.6	SZ/BP		(Maziade *et al.*, 2005)
15	15q26.3	D15S642	100152332	122.1	p = 0.00005	ALC		(Zinn-Justin and Abel, 1999)
17	17q12	D17S1299	36607512	62.0	MLS = 3.6	ASD	male affected sibships	(Cantor *et al.*, 2005)
17	17q21.1	D17S2180	45940225	66.9	MLS = 4.1	ASD	male affected sibships	(Cantor *et al.*, 2005)
17	17q21.32	D17S1290	55583891	82.0	p = 0.00006	ALC		(Bergen *et al.*, 2003)

#: previously named ATP10C; *: genome-wide adjusted.

Locations: cytological locations according to the GMAP estimation of the Genetic Location Database (LDB); NCBI: physical location in nucleotide number according to the National Center for Biological Information; Marshfield: genetic distance from the Marshfield map in centimorgans.

Mental disorders. SZ: schizophrenia; BP: bipolar disorder; SZ/BP: common locus; PBP: psychotic bipolar disorder; ASD: autism spectrum disorder; AS: Asperger syndrome; ADHD: attention-deficit/hyperactivity disorder; ALC: alcoholism.

Statistics. MLS: multipoint Lod score; NPL: non-parametric Lod score; MML: maximum multipoint heterogeneity LOD score; na: not available; LD: linkage disequilibrium; TDT: transmission disequilibrium test; MTDT: multiallelic transmission-disequilibrium test; PDT: pedigree disequilibrium test.

Loci: are those reported for the corresponding markers in the NCBI database. AUTS1, 3, 5: autism 1, 3, 5; ASPG1, 3: Asperger syndrome 1, 3; SCZD7, 9: schizophrenia 7, 9; SCZD10: schizophrenia 10 (periodic catatonia); EJM2: epilepsy, juvenile; MDD2: Major depressive disorder 2; PAND: Panic disorder syndrome; PARK3: Parkinson disease 3.

Genes. KCNN3: calcium-activated potassium channel; PFKFB2: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2; UBE3A: E6-AP ubiquitin-protein ligase; ATP10A: ATPase, Class V, type 10A; GABRB3 GABRA5 GABRG3: gamma-aminobutyric acid (GABA(A)) receptor B3, A5, G3; APBA2: amyloid precursor protein-binding protein; CHRNA7: alpha 7-nicotinic receptor; SLC30A4: zinc transporter; PKM2 (PK3): pyruvate kinase, muscle.

showed also significant linkages with SZ catatonia (Table I), whereas a suggestive linkage (Z = 1,9) was observed on chromosome 22 (Stober *et al.*, 2000b). From these results, it can be observed that ASD shared common susceptibility loci with other mental disorders on five of these chromosomes. On chromosome 1, a susceptibility locus is shared between ASD and SZ at 1q22-q24.2 in a region located between 155 and 165 Mb. The calcium-regulated potassium channel (KCNN3) gene, located at 151,7 Mb slightly centromeric to the shared susceptibility locus, had shown a possible association with SZ but not with a subgroup of SZ showing catatonia (Stober *et al.*, 2000a). Negative association results have also been reported for KCNN3 and both SZ and BP (Glatt *et al.*, 2003b).

On chromosome 7, ASD, SZ, and BP shared a region of 30–40 Mb at 7q31.1-q36.2. The homeogene Engrailed 2 (EN2) gene is located at 7q36. EN2 is specifically involved in patterning the region that gives rise to the cerebellum and controls the plasticity of midbrain dopaminergic neurons. A PvuII polymorphism in EN2 had showed significant differences in the allele frequencies between a sample of 100 autistic children and controls (Petit *et al.*, 1995). Two EN2 intronic SNPs have demonstrated significant association with ASD which supports a neurodevelopmental defect hypothesis in the etiology of ASD (Gharani *et al.*, 2004). However, no significant difference in frequency of a CA repeat polymorphism located in the 3′ region of EN2 was observed between 165 schizophrenic subjects and 97 controls matched for age and ethnicity from a French Caucasian population (Gourion *et al.*, 2004).

On chromosome 13, a linked region of about 30 Mb between 73 and 102 Mb at 13q22.32-q32.3 is shared between ASD, SZ, BP, and psychotic bipolar disorder (PBP) (Table I). The gene D-amino acid oxidase activator (DAOA), previously named G72, is located at 13q34 at the telomeric end of this region (104,9 MB). DAOA had also been associated with SZ in different populations of Caucasians and South-Africans (Hall *et al.*, 2004), of Ashkenazi (Korostishevsky *et al.*, 2004), and of Chinese (Liu *et al.*, 2004; Wang *et al.*, 2004). DAOA has also been associated with BP (Hattori *et al.*, 2003), and with both SZ and BP (Addington *et al.*, 2004; Schumacher *et al.*, 2004). A TDT of a haplotype set including 16 SNPs covering a 157-kb region encompassing the entire complementary DNA sequences of DAOA was significant with BP in a US population (Hattori *et al.*, 2003). This result has been replicated in a second US sample (Chen *et al.*, 2004c), and in Germans (Schumacher *et al.*, 2004). Until located at the extreme end of the 13q22.32-q32.3 susceptibility region and on the opposite side of the ASD potential susceptibility locus, DAOA had also shown a possible association with scores on a premorbid phenotype measured by the Autism Screening Questionnaire (Addington *et al.*, 2004). This region also includes two QTLs: schizophrenia 7 and panic disorder syndrome (Table I). The gene encoding the propionyl Coenzyme A carboxylase alpha polypeptide (PCCA) is located at 13q32 and 99,5 Mb. PCCA, which is expressed both in brain and blood, is involved in the nicotinic acid

metabolism (Van Greevenbroek *et al.*, 2004). It is well known that patients suffering from SZ show an increased prevalence of nicotine addiction which improves, cognitive function affected in SZ patients (Cattapan-Ludewig *et al.*, 2005).

On chromosome 17q12-q21.32 between 37–56 Mb, a susceptibility locus for ASD co-localises with a locus for ALC (Table I). Positive (Conroy *et al.*, 2004; Klauck *et al.*, 1997) and negative (Cook *et al.*, 1997a; Kim *et al.*, 2002; Maestrini *et al.*, 1999; Persico *et al.*, 2000; Zhong *et al.*, 1999) associations with a length and SNP polymorphisms in the serotonin transporter gene located at 17q11.2-q12 and 25,6 Mb, 11 Mb centromeric to the shared susceptibility region, have been reported. A QTL for male serotonin levels was also reported in this region (Weiss *et al.*, 2005a) where the beta 3 integrin gene (ITGB3) is located at 42,7 Mb. Five SNPs in ITGB3 have shown association with the serotonin level in males only, and with a cardiovascular risk factor in females (Weiss *et al.*, 2005b).

Finally, on chromosome 16p13.2 only a close to significant linkage (MLS = 2.93) with ASD was observed for D16S3102 (IMGSAC, 2001a). But this region will be discussed since two positional candidate genes, the glutamate receptor, ionotropic, N-methyl D-aspartate 2A (GRIN2A) and the 4-aminobutyrate aminotransferase (ABAT), both located at 16p13.3, have showed association with ASD (Barnby *et al.*, 2005). This region appeared to be shared with SZ and/or BP (Ekholm *et al.*, 2003; Maziade *et al.*, 2005), and ALC (Detera-Wadleigh, 1999). NMDA channel is involved in long-term potentiation, an activity-dependent increase in the efficiency of synaptic transmission thought to underlie certain kinds of memory and learning, while ABAT is responsible for catabolism of gamma-aminobutyric acid (GABA), an important, mostly inhibitory neurotransmitter in the central nervous system. A variable (GT) (n) repeat in the promoter region of GRIN2A has been associated with SZ (Itokawa *et al.*, 2003b) and BP (Itokawa *et al.*, 2003a), while a GRIN2A exon 5 polymorphism showed association with ADHD (Turic *et al.*, 2004).

2. *Chromosome 15*

Table II presents some of the chromosomal rearrangements and associated syndromes related to ASD for chromosome 15. Most of the rearrangements/abnormalities are observed at 15q11-q13 where the Prader-Willi/Angelman syndrome loci are located. Two more telomeric regions have also been reported with a deletion at 15q22-q23 (Smith *et al.*, 2000), and a duplication of the 15q25-qter region at 15p producing a true trisomy (Bonati *et al.*, 2005). The Figure presents a compendium of all chromosomal rearrangements, linkage results, candidate gene analysis, and assigned susceptibility loci for this chromosome. From these, it could be seen that three to four different chromosomal regions are shared between ASD and other mental disorders (see Fig. 1). The first region is 15q11-q13, located between 19 Mb and 31 Mb, well known to be associated with

TABLE II

SOME OF THE SYNDROMES AND CHROMOSOMAL REARRANGEMENTS RELATED TO AUTISM SYNDROME DISORDER OBSERVED ON CHROMOSOME 15

Location	Syndrome/ rearrangements	Description	References
15p11.2	t(15;16) (p11.2;p13.3) *de novo*		(Martin *et al.*, 2003)
15p11-q15	+der(15)(pter-q15: p11-pter)		(Konstantareas and Homatidis, 1999)
15q11-q12	del(15)(q11q12)		(Schroer *et al.*, 1998)
15q11.2-q13	Prader Willi / Angelman	euchromatic variants, no duplication & clinical features	(Jalal *et al.*, 1994; Ludowese *et al.*, 1991; Mao and Jalal, 2000)
15q11.2-q13	Prader Willi / Angelman	maternal, duplication & clinical features	(Browne *et al.*, 1997; Cook *et al.*, 1997b; Repetto *et al.*, 1998)
15q11.2-q13	Prader Willi / Angelman	paternal, duplication, clinical features or not & obesity	(Cook *et al.*, 1997b; Mao and Jalal, 2000; Mohandas *et al.*, 1999)
15q11.2-q13	dup(15)(q11.2q13)		(Reddy, 2005; Wassink *et al.*, 2001)
15q11.2-q13	del(15)(q11.2q13)		(Wassink *et al.*, 2001)
15q11-q13	dup(15)(q11q13)mat		(Baker *et al.*, 1994; Cook *et al.*, 1997b; Schroer *et al.*, 1998)
15q11-q13	inv dup(15)(q11q13)		(Filipek *et al.*, 2003)
15pter-q13	invdup(15)(pter-q13::q13-pter)mat		(Flejter *et al.*, 1996)
15q22-q23	del(15)(q22-q23)		(Smith *et al.*, 2000)
15q25.2qter	dup(15)(15q25.2qter)		(Bonati *et al.*, 2005)

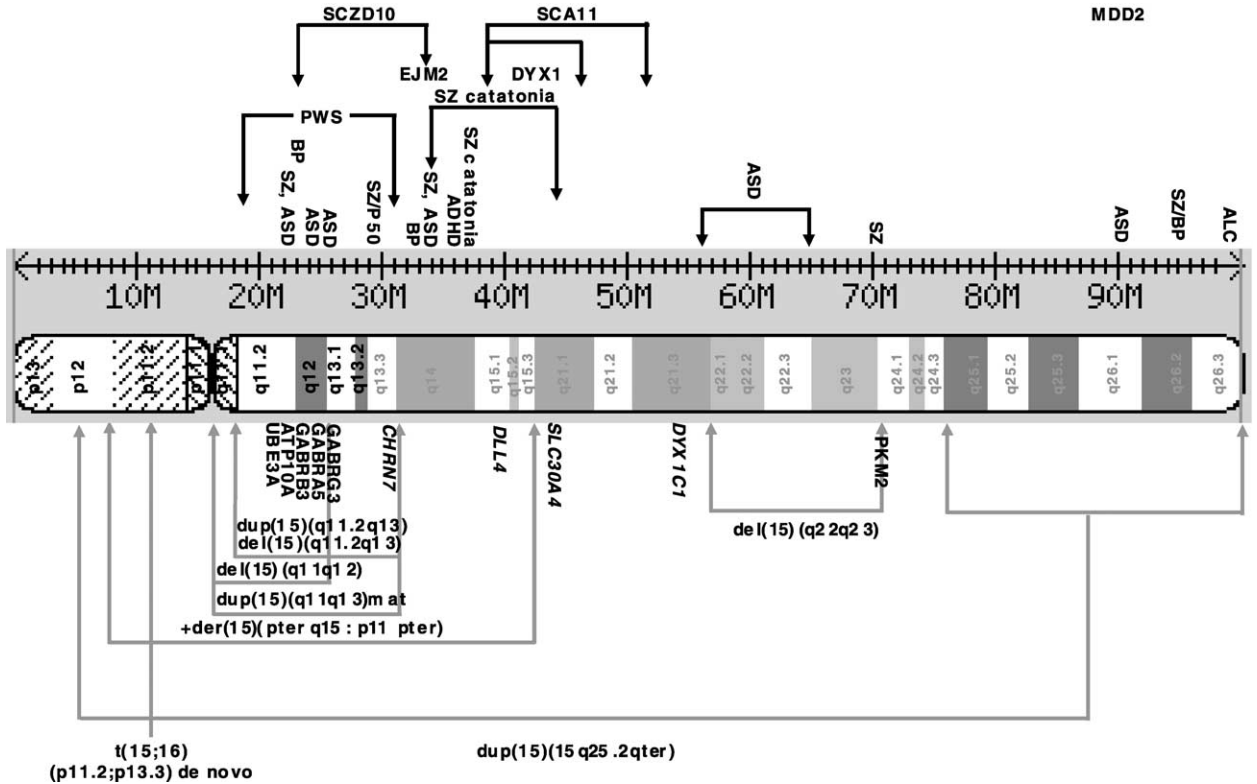

FIG. 1. Linkage results and susceptibility loci (above), and chromosomal rearrangements/abnormalities and candidate genes (below) observed on chromosome 15 in relation to different mental disorders (see text for abreviations). Genes with negative association results are in italic.

ASD because of the numerous chromosomal abnormalities observed in this region in autistic patients (see Table II). Significant linkage and candidate gene association results for ASD have also been observed in this region (Table I). Linkages with the same two markers, both located around 23 Mb, have been observed for ASD and SZ (D15S128), and for ASD and BP (D15S122). The marker D15S122 is located in the 5'UTR region of the E6-AP ubiquitin-protein ligase (UBE3A) gene which has been shown to be associated with the Angelman syndrome (Veenstra-VanderWeele *et al.*, 1999) and ASD (Nurmi *et al.*, 2001). A significant linkage was observed between ASD and the 155CA-2 microsatellite marker located in the gamma-aminobutyric acid (GABA) receptor B3 (GABRB3) (Shao *et al.*, 2003). A positive association with the same GABRB3 155CA-2 marker (Cook *et al.*, 1998), and with two SNPs located in the intron 7 of GABRB3 (McCauley *et al.*, 2004) had also been observed with ASD. In addition to UBEA3 and GABRB3, three other genes located within 1–2 Mb in the 15q11-q13 region have showed an association with ASD: two SNPs and a haplotype block in ATP10A (Nurmi *et al.*, 2003), previously named ATP10C, six SNPs and haplotypes including these SNPs in GABRA5 (McCauley *et al.*, 2004), and two SNPs in GABRG3 (Menold *et al.*, 2001). Also, it is not clear at this time if all these genes are involved individually in ASD, or as a group or subgroups of them interacting with each other in an epistatic effect, or if markers analyzed within these genes are simply in linkage disequilibrium.

A second chromosomal region 15q14-q15.3 located between 32 and 44 Mb is very close to 15q11-q13 and could eventually be part of the same susceptibility region. However, some features could distinguish these two regions. First, most of the chromosomal abnormalities and two susceptibility loci, schizophrenia 10 and the juvenile epilepsy 2, are observed in 15q11-q13 in contrast to 15q14-q15.3 (Fig. 1). Additionally, only the region 15q14-q15.3 shared susceptibility locus with catatonia and ADHD. At 15q14-q15.3, the same marker (D15S118 at 34 Mb) is linked to ASD and SZ, while markers D15S1042, also located at 34 Mb, D15S1012 at 37 Mb, and D15S659 at 44 Mb are linked to catatonia in SZ patients. The zinc transporter gene SLC30A4, located at 15q21.1 and 44 Mb, had shown altered expression patterns in post mortem analysis of the brains of schizophrenic patients (Mirnics *et al.*, 2000). However, no genetic variants within the coding and the putative promoter region of SLC30A4 was found in affected individuals from SZ catatonia large pedigrees showing a perfect co-segregation of a chromosomal segment between marker D15S1042 and D15S659 (Kury *et al.*, 2003). Similarly, no association with catatonia has been observed with the cholinergic receptor, nicotinic, alpha polypeptide 7, and the delta-like 4 (Drosophila) both located at 15q14 and 31,0 and 39,0 Mb, respectively (McKeane *et al.*, 2005; Meyer *et al.*, 2002). Two other QTLs are located in this region: dyslexia-1 (DYX1) and spinocerebellar

ataxia-11 (SCA11) loci. Developmental dyslexia is characterized by an unexpected difficulty in learning to read and write despite adequate intelligence, motivation, and education (Ylisaukko-Oja *et al.*, 2004). The gene dyslexia susceptibility candidate 1 located at 15q21.3 and 53,6 Mb had shown no association with ASD (Ylisaukko-Oja *et al.*, 2004). Autosomal dominant cerebellar ataxia type III is a relatively benign, late-onset, slowly progressive neurological disorder characterized by an uncomplicated cerebellar syndrome. The other spinocerebellar ataxia loci SCA1, located at 6p22.3, has been associated to SZ (Culjkovic *et al.*, 2000; Wang *et al.*, 1996), and it remains to be shown if it could be the same for SCA11. On the other hand, the gene encoding the huntingtin interacting protein K is located at 15q15.3 and 41,9 Mb. The Huntington disease is an autosomal dominant disease that gives rise to progressive, selective, and localized neural cell death associated with choreic movements and dementia.

The third chromosomal region is located at 15q22-q23, between 56 and 70 Mb (Fig. 1). A deletion of this region has been associated with ASD and significant linkage results have been observed for both ASD and SZ. It has to be noted that the linkage with ASD has been observed in non-male affected sib-pairs only which suggests possible parent of origin specific effects (Lamb *et al.*, 2005). One hundred forty genes are located in this region according to NCBI. Among them, the N-methyl-D-aspartate (NMDA) receptor regulated 2 gene (NARG2) is located at 15q22.2 and 58,6 Mb. NMDA receptors play an important role in the transition from proliferation of neuronal precursors to differentiation of neurons (Sugiura *et al.*, 2001). Postmortem brain abnormalities of the glutamate neurotransmitter system have been observed in ASD (Purcell *et al.*, 2001). To our knowledge, NARG2 does not seem to have been studied yet in relation to ASD or SZ. The Bardet-Biedl syndrome 4 gene, characterized by a triallelism and mental retardation, is located slightly outside the shared susceptibility region at 71 Mb, close to the SZ linkage.

The fourth and last region encompassed the q terminal end of the chromosome 15 at 15q25-q26 and 90–100 Mb (Fig. 1). This relatively short region is shared by ASD, SZ, BP, and ALC. Interestingly, the Major depressive disorder 2 susceptibility locus has also been assigned to the same marker (D15S652) linked to ASD. Fifty-five genes are located in this region according to NCBI. The desmuslin gene (DMN) is located at 15q26.3 and 97.5 Mb. DMN is mainly expressed in muscular tissues where it has been involved in different myopathies, but also in brain (Mizuno *et al.*, 2001a,b). DMN encodes an intermediate filament protein that interacts with the desmin, also an intermediate filament protein, and with the alpha form of the dystrobevin (Mizuno *et al.*, 2001b). The beta form of the dystrobevin has been shown to be bound by the dystrobevin binding protein 1 (DTNBP1) located at 6p22.3, and which has been associated to SZ in multiple studies (Funke *et al.*, 2004; Kohn *et al.*, 2004; Schwab

et al., 2003; Straub *et al.*, 2002; VanDenBogaert *et al.*, 2003; van den Oord *et al.*, 2003) and possibly to BP in a subset of cases with psychosis (Raybould *et al.*, 2005). A QTL related to cerebellar ataxia with mental retardation (CAMOS) is also found at 15q26. In skin biopsies of children affected by CAMOS, an inversion of the usual osmiophilic pattern of the vessels is observed and it was thought that this prevented normal exchange between the blood and surrounding tissues, thus decreasing vessel permeability and modifying the production and/or migration of neuronal cells at an early stage (Delague *et al.*, 2002).

3. *Specific Chromosomes*

Finally, susceptibility regions specific to ASD are observed at 2q31.1-q32.1, and at 9p22.3 and 9q34.3. It is not clear at this point if the ASD linkage on chromosome 3 at 3q27.1 is shared or not with SZ and BP since the physical location of the linked marker (D3S3027) is not found in the NCBI database (built 35.1). However, according to the Marshfield genetic map, the ASD linked region is located at more then 30 cM centromeric to the susceptibility regions linked to SZ and BP at 3q29, and the probability that these regions shared the same susceptibility locus is weak. No significant linkage result has been reported for either of the two sexual chromosomes X and Y.

II. Conclusion

Several possible susceptibility chromosomal regions shared between different mental disorders have been identified by putting together on a same physique map scale the significant results from different genome scans. This supports the hypothesis that strong common endophenotype or intermediate phenotypes are shared between these disorders. The genes located in these shared susceptibility regions can help to shed light on metabolic pathways involved and on what could be these common intermediate phenotypes.

Chromosome 15 contains three to four regions shared by five different mental disorders and some interesting candidate genes. As such, this chromosome is a prime target for direct investigations of common intermediate phenotypes in relevant cohorts. Catatonia seems to be a shared syndrome in ASD, SZ, and BP, and may be a useful intermediate phenotype. Findings in this review suggest overlapping susceptibility regions on chromosome 15. Future studies in ASD, SZ, and BP should assess and distinguish the catatonic syndrome within study samples. The identification and validation of additional candidate genes on chromosome 15 by fine mapping and association studies should also be a priority.

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Dirk M. Dhossche

PREFACE

"The most valuable lesson that knowledge can teach us is that its creation depends upon a continuous line of human relationships and traditions that go far back into the past. That continuity is an unbroken thread. It links cultures and peoples; it brings tolerance and understanding; it delivers hope and compassion"

Richard Horton, BSc, MB, FRCP, FMedScie
Editor-in-Chief, The Lancet
The 2004 Elsevier Library Connect Medical Library Lecture

Autism refers to a group of disorders with prominent autistic symptoms, i.e., Autistic Disorder, Asperger Disorder, Childhood Disintegrative Disorder, Rett's Disorder, and Pervasive Developmental Disorder not otherwise specified. These disorders are early-onset behavioral syndromes with a broad range of severity, characterized by lifelong impaired communication, impaired social interactions, and repetitive interests and behavior.

Autism has increasingly come into the limelight. Autism afflicts our young at a much higher rate than the early prevalence studies suggested. The prevalence of the whole spectrum is now considered about 6 per 1000, but some studies suggest it may be as high as 9 or 10 per 1000. Some concern has been raised about a possible increase in prevalence, but changes in diagnostic methodology and ascertainment strategy complicate comparisons across time.

Autism is diagnosed on clinical grounds. There are no diagnostic biomarkers for autistic disorders, except for Rett's Disorder where a causal genetic defect on the X chromosome has been identified. Autism occurs about four times more frequent in males than in females, with an even higher ratio in milder forms. Only in a small proportion of cases can a medical or neurological disorder be found. In the majority of cases, a strong genetic component is suspected, but the pattern of inheritance is complex. Twin studies show a 60% to 91% concordance rate in monozygotic twins, for narrowly and broadly defined phenotypes respectively. In contrast, there are no observations of concordance in dizygotic twins under narrow phenotypic definition; there is 10% concordance under broader phenotypic definition. Sibling recurrence rate is about 4.5%. This pattern of sharply increasing risk for first-degree relatives and monozygotic twins suggests the involvement of multiple genes interacting with one another to lead to disease susceptibility. No definite genetic or environmental causes for autism have been found. Some behavioral and pharmacological treatments have

shown limited benefits in some individuals. No cure for autism has been found yet.

Leo Kanner was the first to describe the core features of autism, in 1943. He reports that the syndrome "differed markedly and uniquely from anything reported so far." Subsequent studies have confirmed the phenotypic validity of autism and have highlighted differences with childhood psychoses and other developmental disorders in cardinal symptoms (e.g., absence of hallucinations in autistic children), course of illness, intellectual functioning, sex distribution, social class, brain abnormalities, age of onset, and family history of schizophrenia. However, boundaries among the different types of autism, and between autism and other disorders, particularly early-onset psychotic disorders and some developmental disorders, are not always clear.

Since Kanner's seminal observations, valuable autism research has been done albeit at the expense of exploring links between autism and other early-onset disorders. It has become increasingly clear that some autistic symptoms may also be present in other disorders that are not in the group of autistic disorders (e.g., childhood schizophrenia), and that some of the molecular events leading to autistic development may not be unique to autism. Research advances in autism and other disorders reveal phenomenological, biochemical, and genetic areas of overlap. The decision to include chapters on schizophrenia, Prader-Willi syndrome, catatonia, and Fetal Alcohol syndrome, collectively referred to as "related disorders" in the title of this volume, acknowledges the importance of exploring areas of overlap between autism and certain non-autistic developmental disorders.

γ-aminobutyric acid (GABA) was discovered in the brain in 1950 by Eugene Roberts. Since then, GABA has risen from obscure brain compound status to stardom. GABA is now considered one of the most important neurotransmitters and developmental signals, with involvement in the pathophysiology of epilepsy, depression, anxiety, alcoholism, and psychosis. Knowledge on the complexity of GABA function has increased exponentially. Undoubtedly, GABA has not revealed all its secrets yet. The actions of GABA are multifaceted and Janus-faced, showing excitation and inhibition at different stages of development. It seems only natural to explore the role of GABA in autism and other developmental disorders.

This volume covers some clinical, but mostly basic research on GABA in the developing brain as it may relate to onset of autism and related developmental disorders. Although the evidence that GABA dysfunction is associated with autism is still limited, findings seem to converge. The chapters, grouped in three sections, are preceded by a comprehensive overview of GABAergic abnormalities in autism by van Kooten *et al.* (Chapter 1)

In the first section "GABA in Early Development" (Chapters 2–5), morphological and functional aspects of GABA signaling in cerebral and cerebellar development are reviewed by Takayama, Jelitai & Madarasz, and Fiszman in their respective

chapters. Terasawa summarizes findings on the importance of GABA function in onset of puberty, a transitional period associated with the emergence and exacerbation of various neuropsychiatric disturbances.

In the second section, "Neurobiology of Autism and Related Disorders" (Chapters 6–14), new findings on common genetic mechanisms in Rett syndrome, Angelman syndrome, and autism are presented by Hogart, Thatcher & LaSalle. Blatt describes abnormalities of the GABAergic cerebellar system in autism. Dhossche, Song & Liu propose GABA hypotheses of autism, Prader-Willi syndrome, and catatonia. Preliminary evidence of plasma GABA as familial marker of autism is presented. Abnormal reelin signaling is reported by Fatemi to be present in both autism and schizophrenia. Rout highlights the role of GABA in prenatal alcohol exposure. The immunological abnormalities in autism are described by Cohly and Panja. Stoppelbein, Sytsma-Jordan & Greening review the psychological and biochemical correlates of abnormal movements in autism. Two chapters by Clement, Pschibul & Schulz, and Welch & Ruggiero examine the role of hypothalamic neuropeptides, including secretin, in autism and related disorders.

In the final section "Genetics of Autism and Related Disorders" (Chapters 15–17), Chagnon examines shared genetic risk factors between autism and major mental disorders. New models of autism, i.e., the GABRB3 and reeler mouse, are presented by D'Arcangelo and DeLorey.

Autism lacks truly effective treatments. The Interagency Autism Coordinating Committee Autism Research Matrix (April 2004, http://www.nimh.nih.gov/autismiacc/congapprcommrep.pdf) that is endorsed by the U.S. National Institutes of Health lists the following goals for achievement within 7–10 years: 1/evidence that 25% of cases of autism can be prevented from symptom expression through early identification and early treatment; 2/methods that allow 90% of individuals with autism to develop speech; 3/the identification of genetic and non-genetic causes of autism (and possible interactions between them), and; 4/the development of efficacious drug treatments that target core symptoms of autism. Three of these four goals concern existing or new treatments.

Some leaders in the field informally predict that new treatments for autism will be found by serendipity, similarly to the discovery of antipsychotic and antidepressant compounds in the 1950s and 1960s. The secretin saga of the last few years offers a salient example. Despite the initial enthusiasm based on a single, but widely publicized, case of dramatic improvement of autism after secretin infusion and a small case-series, large controlled trials have now shown that in most children secretin is not more effective than placebo. Is secretin a dead end? Is secretin only effective in a yet unspecified group of autistic children? Is secretin only effective when administered during a critical phase of autistic development? If so, how much secretin needs to be administered and how often? These questions largely remain unanswered. Arguably, I believe it is still (and possibly even

more) relevant now to find out more about the biochemical and behavioral properties of secretin (and other neuropeptides) as described in Chapters 11 and 12.

Bredero, a famous 16th century Dutch poet, writer, and playwright, signed off on all his work with the phrase "'t kan verkeren" (*things may change*). His motto may also apply to the discovery of new treatments for autism. The history of psychiatry shows us that the discovery of antipsychotics, antidepressants, and other highly effective treatments for severe neuropsychiatric conditions such as electroconvulsive therapy has not led to major discoveries of etiological factors involved in psychotic and affective disorders. Maybe we should be prepared to accept that effective behavioral techniques, drug treatments, or other somatic therapies, might be discovered unexpectedly even if the causes of autism remain as elusive as before. Based on the contributions in this book, I predict that any such treatments will have important effects on GABA. As stated in the foreword by Eugene Robert, the stage may be set for great progress, and Bredero's motto can be honored again: "'t kan verkeren".

Dirk M. Dhossche

FOREWORD

The excellent papers in this volume juxtapose current knowledge of GABAergic function with various areas of knowledge about autism spectrum disorders (ASD) and add much potential building material for the construction of bridges of understanding that eventually may lead to early diagnosis, prevention, and treatments of this dread complex group of diseases. To help round out the picture, one hopes that another volume soon will follow dealing with the glutamatergic excitatory system and the maintenance of the delicate balance between the GABAergic and glutamatergic systems.

Under normal conditions, inhibitory projection and local-circuit neurons play crucial roles in information processing in nervous systems. They are the elements of the nervous system that prevent its lapse into a tyrannical synchronic state of paroxysmal discharge, total inactivity, or chaos by adjusting the timing, sensitivity, and versatility of the processes by which information is received, interpreted, and acted upon. They determine in neuronal assemblies at any particular moment which neurons shall act as groups and which shall act alone and the frequencies and sequences of these activities. Adequate function of the GABA-releasing neurons, the major inhibitory system in the vertebrate nervous system, is essential for maintenance of appropriate information-processing states in various functional modes. The GABA system of inhibitory neurons is a pervasive one and appears to control activities in most areas of the CNS.

The nervous system is highly restrained, inhibitory neurons acting like reins that serve to keep the neuronal ''horses'' from running wild. In coherent behavioral sequences, innate or learned, preprogrammed circuits are *released* to function at various rates and in various combinations. This is accomplished largely by *disinhibition* of pacemaker neurons whose activities are under the dual tonic inhibitory controls of local circuit GABAergic neurons and of GABAergic projection neurons coming from neural command centers. According to this view, disinhibition is permissive, and excitatory input to pacemaker neurons has mainly a modulatory role. Disinhibition, acting in conjunction with intrinsic pacemaker and modulatory glutamatergic inputs is the major organizing principle in nervous system function.

Thus, all activities in nervous systems are critically modulated and shaped by ever-shifting local and global balances between inhibition and excitation, which are largely mediated by the GABAergic and glutmatergic transmitter systems. The spectrum of abnormalities in ASD is remindful of abnormalities that result from gross imbalances between these systems. For example, the deficit in glutamatergic excitatory sensory input produced in normal adults during periods of sensory isolation results in occurrence of hallucinations that are rapidly reversed by return of the individuals to a normal environment. Convulsive seizures in otherwise normal infants with a simple dietary deficiency of pyridoxine (vitamin B_6) was induced by feeding a commercial infant formula from which the vitamin was inadvertently omitted. The seizures were completely eliminated almost immediately after intramuscular injection of pyridoxine because of an extremely rapid conversion of the injected pyridoxine to the co-enzymatically active pyridoxal phosphate, the association of the latter with a suboptimally functional glutamic acid decarboxylase in nerve terminals of inhibitory nerves, accelerated synthesis of GABA therein, and the release of GABA from the terminals onto postsynaptic receptor sites. The consequent desynchronization resulting from the reinstitution of normal neural inhibition prevented the pyroxysmal discharges of groups of neurons whose firing was causing the seizures.

There are many substances that can upregulate or downregulate either GABAergic or glutamatergic transmitter systems. Although a number have been tested in autistic children, none has yet attained the legitimacy of ''standard'' treatment. However, the possibility is not precluded that judicious use of some of these substances administered alone or in combinations together with skillful clinical observation will yield useful treatments in the future.

At this time, I am skeptical about our ability to develop effective, relatively non-toxic ''designer'' drugs for ASD spectrum conditions based on logical extensions of current knowledge of brain neurotransmitter and neuromodulator systems. Rather, I believe that ingenious manipulation of structures and uses of substances arrived at unexpectedly or during empirical screening may lead to therapeutic progress which, in turn, will give rise to new tools for study of basic biological mechanisms.

At one time, it was anticipated that new knowledge of amino acid and vitamin metabolism would lead quickly to the synthesis of analogues of these substances with antibacterial potency. Much effort and money were expended in such endeavors with no success. Instead, the ''antibiotic revolution'' stemmed from the concerted exploitation of an observation that a

mold contaminating a bacterial culture caused bacteria in its vicinity to undergo lysis and that the broth in which the fungus was grown was bacteriocidal for many common pathogenic organisms. The mechanism of action of penicillin was elucidated only many years after millions of lives had been saved by its use.

Most drugs effective in treatment of nervous system disorders arose from the folk medicine of ancient civilizations, from empirical searches among many substances for specific desired effects, or from unexpected observations. For example, the anticonvulsant properties of bromide and phenobarbital were discovered by chance. The development of diphenylhydantoin (Dilantin) for treatment of seizures resulted from a planned search for compounds capable of suppressing electroshock convulsions in laboratory animals while not showing the degree of sedation or impairment of consciousness seen with barbiturates. Meprobamate (Miltown) was selected as an anti-anxiety drug from tests of over 1200 compounds. The first benzodiazepine to be used clinically, chlordiazepoxide (Librium), was noted to have a ''taming'' effect in several species of animals. This led to the trial of the drug for anti-anxiety effects and to the synthesis of diazepam (Valium) and a number of other benzodiazepines that currently are used in treatment of anxiety and tension, sleep disorders, epilepsy, muscle spasms and a variety of so-called psychosomatic disorders. It was approximately 20 years after initiation of clinical use of benzodiazepines that one of the mechanisms of their action was linked to function of the receptor-chloride-ionophore system regulated by GABA, thereby sparking great progress in our understanding of that system. Barbiturates, benzodiazepines, bromide, and ethanol were found to converge in their actions at the level of the GABA receptor complex, leading to further understanding of its components and their relationships.

Although for some time there has been much information about the glutamatergic and GABAergic transmitter systems, respectively, few widely clinically useful drugs based on this knowledge have been devised to date. On the other hand, curiosity about the analgesic and anti-spastic properties of Baclofen, a failed GABA-mimetic in classical Cl^- channel conductance paradigms, led to the discovery of $GABA_B$ receptors, which are coupled to K^+ channels and to many new therapeutic possibilities.

Over the years I have engaged in studies related to problems of epilepsy, schizophrenia, Huntington's disease, senile dementia of the Alzheimer's type, hepatic coma, multiple sclerosis, and spinal cord injury. After perusing the papers which will appear in this volume and surveying the current literature, I am adding ASD to the list of my concerns and hope that

among the other readers of this material there will be some who do likewise. The stage may be set for great progress.

Eugene Roberts, Ph.D., FAIC
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The Reeler Mouse: Anatomy of a Mutant

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Shared Chromosomal Susceptibility Regions Between Autism and Other Mental Disorders

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