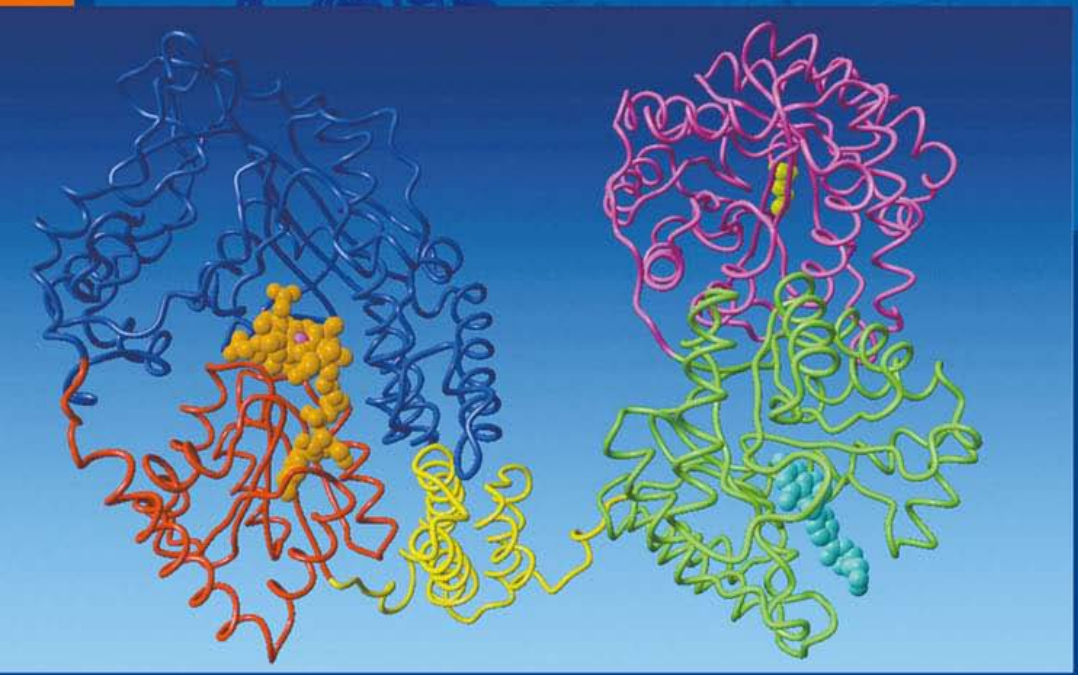
Gene J. Blatt
Editor

The Neurochemical Basis of Autism

From Molecules to Minicolumns



Springer

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To my wife Faith and my family for their love and support and to families of individuals with autism for their strength, perseverance, and unwavering desire to find answers.

Preface

With the recently perceived increase in incidence of autism and the realization that "autism" may actually be "autisms" with subsets of affected individuals, researchers have been pursuing the possibility that there may be multiple etiologies for the disorder. Although most autism studies have focused on genetics and advanced neuroimaging, there is a paucity of research aimed at determining the neurochemical basis of autism. Identifying core neural substrates or key biomarkers is essential to understanding the mechanistic basis that may in part underlie "autisms." Alterations in molecules, proteins, receptors, and synaptic elements are some of the contributing substrates that could result in altered developmental processes, changed synaptic function, and aberrations in connectivity. It is now apparent that multiple brain areas are affected in autism, and neuropathological defects have been described within cortical and subcortical networks. Although recent progress has been made in identifying some of the genes that may underlie the disorder, much attention has also been given to epigenetic and/or environmental factors that may contribute to subsets of autistic individuals.

The contributors to this book were hand selected because of their expertise in their respective fields. Individually each chapter presents a unique perspective into the clinical, developmental, neurochemical, and/or physical chemical basis of autism. The contributing authors summarize current research findings in their respective areas and also present novel ideas and propose hypotheses and possible mechanisms that may be operative during development and the potential consequences of having defects in specific molecules, receptors, or genes.

The subtitle "From Molecules to Minicolumns" was inserted because of much recent attention given to alterations in the basic organization of mini- or microcolumns of neurons in cerebral cortical areas in autism. These especially include prefrontal cortical areas that undergo an overgrowth during early postnatal development in many individuals with autism. To this end, the world renowned Dr. Alan Peters, the neuroanatomist that originally described mini- or micro-columnar organization in the cerebral cortex, was recruited to write a chapter in this book giving his expert perspective on the issue in autism.

The book begins with highly respected clinician, Dr. Margaret L. Bauman, Director of the LADDERS clinic in the Boston area, with a clinical and medical perspective of autism discussing etiologies, clinical presentation, early identification,

advancements in medical care, and associated disorders. In the chapter "The Male Prevalence in Autism Spectrum Disorders: Hypotheses on its Neurobiological Basis", Italian researchers Drs. Flavio Keller and Liliana Ruta present neurochemical hypotheses as the basis for the predominance of male prevalence in autism discussing the possible roles of estrogen, testosterone, oxytocin, and vasopressin in the organization of brain circuits and hemispheric specialization. Psychiatrist Dr. Ricardo Vella relates neuropathologies in autism, in the limbic and cerebellar regions, to specific behaviors and presents a developmental perspective and hypotheses regarding emotional and attachment behaviors in autistic individuals. The chapter "The Morphology of Minicolumns" continues on the neuropathology theme by the aforementioned Dr. Alan Peters, an intensive review on normal minicolumn organization and how it is altered in normal aging, Alzheimer's disease and autism. This is essential reading to understand the basic structural and functional unit of cortical organization and how it is affected in neurobiological disease states.

The chapter "The Developmental Neuropathology of Autism" contributed by the well-recognized neuropathologist, Dr. Thomas Kemper, relates neuropathological changes in autism to the pre- and postnatal developmental timing of the disorder. Defects in cellular pathology such as abnormal cell size, ectopic neurons, decreased numbers of neurons, and/or possible myelination defects are related to abnormal patterns of brain growth and developmental timing in autism. Neurochemical defects during development is the theme of the next chapter contributed by Dr. Diane Chugani discussing using positron emission tomography (PET) molecular imaging providing information regarding time course differences in the ontogeny of various neurochemical processes in children with autism. Dr. Chugani describes how developmental changes in serotonin synthesis and $GABA_A$ receptor binding in children are important in developing new therapies during critical developmental windows.

The chapter "Glutamic Acid Decarboxylase (GAD) as a Biomarker of GABAergic Activity in Autism: Impact on Cerebellar Circuitry and Function", contributed by the Editor and colleagues, focuses on changes in the cerebellum in autism and how alterations in mRNA in key synthesizing enzymes for GABA (GAD65 and GAD67) underlie defective circuitry with potential consequences for output projections to thalamic, cortical, and/or subcortical regions and the effect on motor and/or cognitively based behaviors. With all the recent attention on chromosomal defects in autism such as duplications/deletions in chromosome 15q11–13 region that contains three $GABA_A$ receptor subunit genes, Dr. Amber Hogart and renowned researcher Dr. Janine LaSalle present the chapter "Epigenetic dysregulation of 15q11-13 $GABA_A$ Receptor Genes in Autism" on epigenetic dysregulation of gene effects on this region in autism and in a variety of neurodevelopmental disorders. Drs. Mukaetova-Ladinska, Westwood, and Perry and in chapter "Cholinergic Component of Autism Spectrum Disorder" describe changes in muscarinic and nicotinic cholinergic receptor changes in autism brain areas in children and adults. The authors also discuss the use of cholinesterase inhibitors and receptor antagonists as intervention therapies for treatment of cognitive and non-cognitive behavior changes in autism spectrum disorders (ASDs). The chapter "Oxytocin and Autism" revisits the role of oxytocin in autism focusing on its role in social behavior.

Drs. Peter Kirsch and Andreas Meyer-Lindenberg from Mannheim, Germany, are experts on the prosocial neuropeptide oxytocin and discuss its role in humans and its relevance for autism pathogenesis and therapy. In the chapter "The Role of the Noradrenergic System in Autism Spectrum Disorders", Dr. David Beversdorf presents the normal role of norepinephrine and its effects on cognition and the possible dysregulation of norepinephrine in autism and possible treatment with propanolol.

Dr. Richard Deth and colleagues in the chapter "Oxidative Stress in Autism and Its Implications for Dopamine-Stimulated Phospholipid Methylation" discuss the relationship of oxidative stress and autism. Impaired methylation is a consequence of oxidative stress, and the authors present a discussion of how metabolic events contribute to impaired methylation and the role of dopamine D4 receptor activation in gamma frequency synchronization of neural networks during attention which is thought to be defective in autistic children. At the synaptic level, Drs. Craig Powell and Antony Boucard discuss the important topic of mutational defects in specific types of postsynaptic neuroligin–3 and –4 linked to the presynaptic cell adhesion molecule neurexin–1 affecting trans-synaptic bridges in rare cases of autism in the chapter "Neuroligins and Neurexins: Synaptic Bridges Implicated in Autism". The authors describe in detail the mechanisms that underlie such defects and present an animal model and its effectiveness. Perhaps the most innovative chapter is the one presented by Dr. Peter Bergathon, a neurologist, physicist, and physical chemist who is an expert in neuroscience systems "intelligence modeling" and applies its principles to develop a novel hypothesis based on the energy demands of certain types of computational strategies in the brains of individuals with autism. The energetics of information transfer in the autistic knowledge surfaces for solving system analysis problems including language and reciprocity are unfavorable compared to manifolds associated with more natural behaviors. Some may find this fascinating treatise challenging, but Dr. Bergathon's analysis suggests that autistic behaviors may be the result of an attempt to manage a highly unfavorable energy cost when cognitive dynamical processes are demanded from a neural system ill suited for these tasks.

In the final chapter, an expert pharmacologist, Dr. Terrell Gibbs presents a comprehensive review of pharmacotherapies in autism. He details their results from clinical trials, their effectiveness, and their role in the treatment of autistic behaviors. Special emphasis is given to the atypical antipsychotic drug risperidone that is frequently effective for ameliorating symptoms of irritability, hyperactivity, social withdrawal, and stereotypic, repetitive behavior in autism.

In summary, this book presents a fresh perspective on some of the groundbreaking research and novel hypotheses being applied to the neurochemical, developmental, and physiochemical etiologies and treatments of autism. Included is an Appendix with lay summaries of all chapters in the book to help the educated lay individual in understanding these presentations by experts in the field. The book is aimed at contributing to the understanding of autism as well as advancing our knowledge in developing effective pharmacotherapies. The hope is that with continued efforts and contributions from the scientific community, individuals with autism

will find effective and treatment improvements in their lives. The authors and editor would like to thank the families for their unending campaign to support research efforts and raise awareness as well as their generous donations of brain tissue for post-mortem studies.

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Autism Spectrum Disorders: Clinical and Medical Perspectives

Margaret L. Bauman

Introduction

Autism is a behaviorally defined disorder, first described by Kanner in 1943. The following year, Hans Asperger published a report of four children with "autistic psychopathy" (Asperger, 1944). Since that time, the definition of autism has evolved from the very narrow view of "early infantile autism" to an expanded and more detailed description as delineated in the Diagnostic and Statistical Manual (DSM-IV) (l994). The current classification includes autism within the broader category of pervasive developmental disorder (PDD) along with Asperger's syndrome, Rett's Syndrome, childhood disintegrative disorder and pervasive developmental disorder not otherwise specified (PDD-NOS). However, as more clinical investigations and basic science research have become increasingly available over the past several years, it has become apparent that autism involves a continuum of severity and symptoms, and as a result, the term "autism spectrum disorders" (ASD) has come into common usage.

Etiology

Autism is now considered one of the most common disorders of development worldwide. Studies performed by the United States Centers for Disease Control (CDC) in selected communities in 2002 suggest that the current prevalence rates for ASD are approximately 1 in 150 children (Kuehn, 2007). The increase in the reported prevalence of ASD has been attributed by some to improved ascertainment, a broadening of the diagnostic definition, and improved public and professional

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awareness. Others have attributed this upsurge in diagnosis to the contribution of potential, as yet to be identified, environmental factors.

Numerous epidemiologic studies have provided compelling evidence for a genetic basis for autism (Bailey et al., 1995; Bolton et al., 1994). Beginning with the seminal twin study of Folstein and Rutter, published in 1977, the concept of ASD as a largely genetic disorder has remained in the forefront of autism research. In this study, the authors identified a higher concordance in monozygotic twins than in dizygotic twins. Since that time, numerous linkage studies have been reported with the most frequently replicated findings being associated with chromosomes 7q, 15q, 22q, and 2q (Schaefer and Mendelsohn, 2008). Additional candidate genes of promise include GABA (gamma amino butyric acid), serotonin transporter genes, Engrailed 2, Neuroligin, MECP2, WNT2, PTEN, and MET (Campbell et al., 2007). Autism is four times more common in males than in females, with a higher ratio in milder forms of the disorder. Further, ASD is associated with a significant familial recurrence, much higher than that seen in the general population. The reported recurrence risk has been estimated to be approximately 15% in families having one affected child (Landa, 2008; Landa and Garrett-Mayer, 2006; Lauritsen et al., 2005). If the family has two affected children, the recurrence rate for subsequent children increases substantially, up to 25–50% (Cook, 2001; Spence, 2004).

Despite modern technology and advanced research, only approximately 6–15% of individuals with autism will be found to have an identifiable genetic diagnosis. In addition, a number of syndromes have been associated with ASD including Fragile X syndrome, Tuberous Sclerosis, Smith–Lemli–Opitz syndrome and Rett syndrome (MECP2 mutations) (Schaefer et al., 2008). Numerous genes have been investigated as possible candidate genes, but replicated findings are lacking. Current epidemiological studies of ASD strongly suggest multifactorial inheritance, including genetic heterogeneity with multiple major gene effects, possibly contributing environmental effects and physiologically linked processes with multiple genes.

One of the many additional potentially important risk factors for ASD that has gained increased interest is the role of advance parental age. In a recent study, Durkin et al. (2008) noted that in a study of 1251 children with complete parental age information and who were defined as having ASD based on DSM-IV criteria, both maternal and paternal age were independently associated with autism. The authors also noted that firstborn offspring of two older parents were three times more likely to develop autism than were later born offspring. A number of potential mechanisms for these effects have been suggested including age-related chromosomal changes, complications of pregnancy, or possible environmental exposures during pregnancy that could have mutagenic effects. In addition, given the apparent importance of birth order, the authors speculate that these children may be more susceptible to autoimmune responses affecting neurodevelopment or may be affected secondary to maternal exposure to neurotoxic chemicals, passed to the offspring transplacentally or in breast milk, in combination with advanced maternal age. Whatever the mechanisms involved, these observations warrant further investigation in a larger population of ASD children.

Clinical Presentation

Although it is now recognized that autism is a clinically and biologically heterogeneous disorder, those affected share a triad of common features which include atypical social interaction, delayed and disordered language, and a markedly restricted repertoire of activities and interests (American Psychiatric Association, 1994). Symptoms can range from relatively subtle and mild to very severe. For example, there may be a qualitative impairment in reciprocal social interaction as opposed to an absolute absence of social interaction. Social behaviors can range from a seemingly total lack of awareness of others to inappropriate eye contact and atypical social responsiveness. Communication skills can span from a total lack of verbal speech and intentional use of gesture to the presence of speech that is associated with atypical intonation, prosody, syntax, and grammar. Although the normal development of single word receptive and expressive vocabulary may be present, pragmatic language may be significantly impaired. Many affected individuals demonstrate poor eye contact, echolalia, pronoun reversals, stereotypic and repetitive behaviors, sensory processing dysfunction, difficulty dealing with novelty, and an obsessive reliance on routine and some level of cognitive impairment. In very young children, the lack of a pointing response and joint attention and limited pretend play are frequent characteristics as early as 12 months of age. Many affected individuals have exceptional islands of rote memory and outstanding isolated talents in the face of otherwise general functional disabilities. Although it was initially believed that approximately 75% of those affected with autism functioned in the mentally retarded range, more recent studies have found that fewer than half of affected individuals have significant cognitive impairment (Newschaffer et al., 2007).

Typically, those individuals with non-syndromic or "essential" autism demonstrate few if any dysmorphic features and are generally described as very attractive appearing children. For many years, these children were believed to demonstrate no abnormalities of motor function or if present, these deficits were believed to be merely associated symptoms. It has now become apparent that gross and fine motor dysfunction is more common than previously appreciated. Numerous clinical studies indicate that children with autism exhibit deficits in skilled motor performance in response to command and with tool use, suggestive of a more generalized dyspraxia (Rogers et al., 1996; Mostofsky et al., 2006). Children with autism often show delays in learning novel complex motor skills such as peddling a tricycle or pumping on a swing with their legs (Gidley Larson and Mostofsky, 2006). Further, in a study of motor impairment in a group of 154 ASD children, Ming et al. (2007) noted that hypotonia was the most common motor symptom in this cohort, with motor dyspraxia being more prevalent in younger children than older children. Gross motor delay was reported in 9% and toe walking in 19%. The etiology of motor dysfunction in ASD remains uncertain with abnormalities of the cerebellum, basal ganglia, and/or neural connections across distributed networks being hypothesized (Gidley Larson et al., 2008).

The role of the clinician is to try to identify specific etiologic factors that may contribute to the diagnosis of ASD and then to provide appropriate guidance and counseling based on the information obtained. Frequently, the parent is the first

to raise concerns about their child's development with their medical provider and these concerns need to be taken seriously by the health-care community. Despite significant advances in basic science and clinical research, the diagnosis of ASD remains largely a clinical one, based largely on behavioral history and developmental assessments and observation. Although not typically used on a routine basis by primary care physicians, formalized internationally accepted measures such as the Autism Diagnosis Interview-Revised (ADI-R) and the Autism Diagnostic Observation Schedule-Generic (ADOS-G) are used in many specialty clinics and research programs and have resulted in a greater reliability in diagnosis (Lord et al., 1994, 2000). According to its current definition, the clinical features of autism typically become evident before 3 years of age and are usually associated with varying degrees of developmental delay.

For the average primary care physician, the ADI-R and ADOS-G are too time intensive to administer in a busy pediatric and family medicine practice. However, there are now a number of screening tools and parent questionnaires available that can be used. Although not considered to be adequate to make a definitive diagnosis, these tools can identify potential risk factors and clinical signals that should then lead to a referral for further assessment.

Examination of the autistic child, adolescent, and adult may be complicated by variable levels of cooperation, impaired communication, and behavioral issues. Important factors during the physical and neurological assessments should include identification of potential dysmorphic features that might suggest a specific diagnosis or syndrome. Measurements of head circumference throughout childhood has resulted in the observation that a subset of ASD children demonstrate a larger than average head circumference, with approximately 20% of these showing a frank macrocephaly of greater than 98% for age and sex. More recent work by Lainhart et al. (2006) highlight the fact that the distribution curve of head size in ASD is similar to that seen in typically developing children but is shifted to the left, suggesting that this unusual head growth may reflect an up-regulation of as yet unknown growth factors. The clinical finding of macrocrania is, at this time, without a defined neuropathological correlate.

All patients with autism should have a formal audiogram. Many ASD children present with impaired receptive and expressive language and fail to respond to the spoken word, causing some parents to wonder if their child might be deaf. Impaired hearing could alter communication and socialization skills. There is a debate as to the role of electroencephalography (EEG) as part of the routine evaluation of a child with ASD. Although there are reports of autistic-like symptoms in some children with seizure disorders and an acquired aphasia (Landau–Kleffner Syndrome), this disorder is very rare. In general, EEG is probably not indicated as a routine part of the ASD evaluation unless there is a clinical history to suggest a possible seizure disorder. Similarly, cranial imaging studies are not routinely recommended unless abnormalities on neurological examination are observed (Filipek et al., 1999). Additional assessments should include high resolution karyotype and Fragile X studies as an initial step, along with assessments from a speech and language pathologist, an occupational therapist, and a cognitive developmental specialist.

Early Identification

Given the observation that early intervention services can have a positive impact on developmental outcome in ASD, a growing number of research studies have been initiated with the goal of identifying the earliest possible clinical indicators for the disorder. One of the earliest such study involved the review of first birthday video tapes in children who later were diagnosed with ASD in comparison with those of typically developing children (Osterling and Dawson, 1994). The investigators noted that, at 12 months of age, the ASD children did not use a pointing response for communication purposes and failed to show consistent joint attention. Because of the known risk of having another child with autism in families having one affected child, more recent research has involved prospective investigations of infant ASD siblings, studied in comparison with age- and sex-matched siblings of typically developing children. In general, diagnostic assessments have focused on the development of behavioral, cognitive, and communication markers beginning at 6 months of age, although some are beginning to evaluate high-risk mothers, follow them through pregnancy, and beginning with the observation of the child immediately at or after birth.

Studies of infant siblings have provided some clinical markers that appear to be associated with a later diagnosis of autism. Although most of these studies fail to show significant differences that are reliably diagnostic before the age of 12 months, some studies have highlighted subtle abnormalities in social engagement (Bryson et al., 2007). Other diagnostic features have included delayed development in verbal and non-verbal communication (receptive and expressive language, gesturing, pointing, showing), imitation, pretend play, response to name, visual attention to objects and social interaction (Landa, 2008). Thus, it is now possible to diagnose autism with reasonable assurance by the age of 2 years in many cases and it is anticipated that with continued research involving still younger infant autism siblings, earlier diagnosis may yet still become a reality.

Currently, there are no reliable biomarkers to identify those at risk for autism. However, since 2001, there has been the increasing appreciation that a substantial subset of ASD children have been found to have an up-regulation in head growth, most evident during the first 2 years of life, plateauing off by 16 years of age (Lainhart et al., 2006). The cause of this early brain enlargement is unknown although a number of biologic mechanisms have been suggested including failure of synaptic pruning, neuronal loss, or white matter overgrowth.

Associated Medical Disorders

Until recently, much of the clinical and basic science research has been focused on the understanding of brain mechanisms that could explain the behavioral, cognitive, social interaction and communication dysfunction associated with ASD, with relatively little attention to the possible significance of accompanying medical conditions. Much of this relative neglect may be related to the fact that the physical

examination of an individual with autism, particularly children, can be challenging and often limited by poor patient cooperation and difficult office behavior. Further, it now appears that ASD individuals, many of whom are non-verbal or hypo-verbal, may not be able to describe or localize their discomfort. In addition, there is a growing appreciation that ASD persons may not present with typical, easily recognized symptoms, making diagnosis in any one circumstance difficult, and therefore overlooked. Research indicates that children with ASD are more likely than other children with special needs to have difficulty accessing medical care, further compounding the challenge of providing quality routine health care to this population of individuals (Kogan et al., 2008). The fact that a child has autism does not rule out the possibility that he/she may have one or more other illnesses or disorders, similar to those experienced by typically developing children. Identifying and treating these disorders may improve behavior, developmental progress, and quality of life, as well as provide leads into potential subsets of ASD individuals that may have genetic and etiologic implications. Space does not allow for a detailed description of the multiplicity of possible medical conditions that may affect a person with autism. Therefore, only some of the more common disorders will be briefly highlighted here. These include seizure disorders, sleep disturbances, gastrointestinal disorders, metabolic dysfunction, and hormonal imbalances. However, the primary care and specialty physicians serving ASD persons must be constantly alert to a wide range of medical possibilities at any one time.

Seizure Disorders

The prevalence of seizures in adults with autism has been estimated to be between 20 and 35% (Minshew et al., 1997), and in ASD children between 7 and 14% (Rapin, 1996; Tuchman et al., 1991), with peak risk periods occurring in early childhood and adolescence (Volkmar and Nelson, 1990). Although regression of language and cognitive skills in association with seizures has been reported during the teenage years, little is known regarding its etiology or prevalence (Minshew et al., 1997). Seizures may be of any type but with partial complex seizures being most frequently reported. The clinical identification of partial complex seizures in autistic individuals can often be complicated by the presence of atypical behavioral patterns and body movements often seen in association with ASD and frequently attributed to the autism per se. Alternatively, not all body movements or mannerisms observed in ASD are seizure related and may be manifestations of other medical conditions such as gastroesophageal reflux disease (GERD) (Buie, 2005). Further complicating diagnosis is the fact that there may be a lack of direct correlation between clinical seizures and electroencephalographic (EEG) activity (Minshew et al., l997). However, any behaviors such as staring, cessation of activity, eye fluttering, or aggressive behavior associated with confusion should raise the suspicion of a complex partial seizure and further evaluation pursued. The obtaining of a high-quality

EEG, especially in toddlers and young children can be difficult but can be achievable. Prolonged or overnight EEG can often be helpful as well as the use of video tape review of the events recorded in the home or school. A growing number of anticonvulsant medications are now available and these seizures can usually be brought under control with experienced medical management.

Gastrointestinal Disorders

Although gastrointestinal (GI) dysfunction is believed to be relatively common in autism, the true prevalence of these disorders is unknown (Buie, 2005; Campbell et al., 2009). Similarly, it is not known whether or not these disorders are more common in persons with ASD than in typically developing individuals. However, a recent, well-controlled prospective study, using a structured interview, reported a significantly increased prevalence of GI conditions in ASD as compared to controls (Valicenti-McDermott et al., 2006). Parents often report a number of symptoms in their babies and young children, including diarrhea, constipation, food intolerance, gas, bloating, abdominal pain/discomfort, and a history of reflux (Horvath et al., 1999; Quigley and Hurley, 2000).

Although many ASD children present with typical GI tract symptoms, others may not, and may instead, exhibit aggressions and self-injurious behavior (SIB), facial grimacing, chest tapping, and the seeking of abdominal pressure (Buie, personal communication). It is well known that typically developing children can and often do present with behavioral disruptions when not feeling well. There is no reason to believe that ASD children should do otherwise. Gastroesophageal reflux disease (GERD), gastritis, esophagitis, colitis, inflammatory bowel disease, Crohn's disease, and celiac disease have been identified in autistic persons and treatment of these disorders has resulted in improved behavior and better developmental progress (Buie, personal communication), no doubt because the individual is more comfortable and physically well.

Recently, Campbell et al. (2009) have reported that disrupted MET gene signaling may contribute to increased risk for autism spectrum disorder that includes familial GI dysfunction. A functional variant in the promoter of the gene encoding the MET receptor tyrosine kinase has been associated with ASD, and MET protein expression has been found to be decreased in the temporal lobe cortex in ASD postmortem brain tissue. MET is a pleiotropic receptor that is known to function in both brain development and GI repair. Thus, the identification of medical disorders in ASD individuals, in this case GI disorders, may not only improve quality of life for those affected with ASD but may lead to improved or more precise definition of genetic and phenotypic subtypes in this complex heterogeneous disorder.

Sleep Disorders

The prevalence of sleep disorders in typically developing children is said to be approximately 30% and appear to be more common in early childhood (Ferber,

1996). In contrast, the prevalence rates among children with autism have been estimated to range from 44 to 83% (Richdale, 1999), and sleep disorders have been reported to be more severe in this population (Malow et al., 2006). It is known that disordered sleep affects daytime health, neurocognitive dysfunction, and behavioral disruptions in a variety of psychiatric and neurologic conditions. In typically developing children, sleep disruption may lead to daytime sleepiness and may manifest itself as hyperactivity, inattention, or aggression (Owens et al., 1998). Insomnia, defined has having trouble initiating and/or maintaining sleep, is the most common feature reported by parents of autistic children. Additional sleep concerns include symptoms of disordered breathing associated with loud snoring, noisy breathing, or occasional pauses or apneas in breathing as well as leg movements and tooth grinding. Occasionally, nocturnal arousals associated with screaming, walking, or confusion has been reported. These sleep disturbances may have multiple causes. Trouble initiating sleep may be related to hyperactivity or medications used to treat hyperactivity. Anxiety, depression, seizure disorders, or abnormalities of circadian rhythm have also been associated with delayed sleep onset.

While disorders of arousal and behavioral non-compliance may also be factors, one must also consider other medically based disorders. It is known, for example, that gastroesophageal (GE) reflux can contribute to night time awakenings in infants as well as in older children (Buie, unpublished data). Loud snoring and daytime mouth breathing may suggest enlarged tonsils and/or adenoids as possible factors (Owens et al., 2000). Further, both obstructive and central sleep apnea have also been reported in some cases. Given that there is increasing evidence that poor quality and quantity of sleep can have a negative effect on day time behavior and functioning (Malow et al., 2006), It is important to accurately diagnose and treat reported sleep disruptions in individuals with ASD.

Metabolic Disorders

Metabolic disorders are considered a rarity among patients with neurodevelopmental disorders, a reported diagnostic yield after initial evaluation varying from 1 to 2.5% (van Karnebeck et al., 2005). Although rare, the effect of correct diagnosis and treatment of a metabolic disorder may have a substantial effect on the patient's developmental outcome. Engbers et al. (2008) performed repeated metabolic studies on a series of 433 subjects with neurodevelopmental disorders whose initial metabolic assessments were said to be normal and identified 12 metabolic diseases (2.8%), some of which were treatable. The prevalence of metabolic disorders in ASD remains as yet largely unknown with the level of frequency no doubt varying with the specific disorder.

A number of studies and case reports have suggested that mitochondrial disorders may be a causative factor in a subset of autistic individuals. In 2005, Oliveira et al. published a population-based survey among school-aged children with ASD and found that 7% of those who underwent a complete metabolic evaluation were

diagnosed with a mitochondrial respiratory chain disorder. Further, this group reported that the affected children were clinically indistinguishable from ASD children without a mitochondrial dysfunction. More recently, however, Weissman et al., (2008) reported their findings on 25 ASD subjects with biopsy-proven mitochondrial disorder and found that a series of "clinical red flags" could distinguish the affected children from those with idiopathic ASD. These "red flag" characteristics included the involvement of at least one non-neurological organ system, significant gross motor delays, easy fatigability, and repeated regressions after 3 years of age. Further, they noted a nearly even distribution between males and females. The authors concluded that with careful clinical and biochemical assessments, children with the co-occurrence of ASD and mitochondrial dysfunction could be distinguished from those with idiopathic ASD and that those affected with mitochondrial disorders may represent a significant subset of individuals with autism.

Other medical conditions that have not yet been well studied appear to include the potential effects of hormonal imbalance, especially during adolescence, which could be associated with precocious puberty, accelerated or reduced physical growth, and/or behavioral disruptions associated with menstrual pain or discomfort. In addition, the presence of recurrent ear infections, sinusitis, hearing impairment, hypertrophied tonsils and adenoids, urinary tract infections, spastic bladder that to be associated with new onset incontinence at any age, attention deficit disorder, disordered sensory processing, allergies, or any other medical condition commonly seen in typically developing children should be considered in ASD individuals at any age. Defining and treating these medical conditions can improve quality of life for the patient as well as his/her family and can be associated with improved developmental gains as the result of better physical health.

Conclusion

Much progress has been made in the identification and treatment of persons with ASD, as well as our understanding of the etiologic and biological mechanisms that are or can be associated with the disorder. However, much still remains to be unraveled and many questions remain unanswered. With advancing technology and improved medical and diagnostic assessments of those affected with ASD and their families, it is hoped that diagnoses can be made earlier, potentially at or before birth, and that more effective therapies and interventions will become possible to improve the lives of those on the spectrum.

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The Male Prevalence in Autism Spectrum Disorders: Hypotheses on its Neurobiological Basis

Flavio Keller and Liliana Ruta

Introduction

Autism and the related disorders Pervasive Developmental Disorder Not Otherwise Specified (PDD NOS) and Asperger syndrome (AS) are neurodevelopmental conditions with strong heritability (Muhle et al., 2004) characterized by impairment in social-communicative skills and a restricted and repetitive pattern of interests and behaviours. Together they are broadly referred to as autism spectrum disorders (ASD) (APA, 2000).

The widely accepted concept of a "spectrum" properly emphasizes the variability in the phenotypic characteristics within this condition (Volkmar et al., 2005) and opens a relevant debate on how to consider the clinical heterogeneity of the autistic core symptoms in terms of social impairment, communication deficit and restricted/repetitive behaviour.

The main two theories that addressed this issue are referred to as the "subgroup model" and the "severity model".

According to the subgroup model this variation might be explained in terms of discrete phenotypes within the disorder with unique, even if partially overlapping, gene contributions to the different aspects of the phenotype such as social interaction, language and interests (Silverman et al., 2002; Ronald et al., 2006; Shao et al., 2003).

On the other hand, the severity model assigns the different presentation of the core symptoms observed in autism to a continuous severity gradient with an additive genetic effect in terms of susceptibility – i.e. the more susceptibility genes a person has, the more severe the phenotype will be (Spiker et al., 2002; Constantino et al., 2004; Ring et al., 2008).

Unfortunately, although several twin and family studies (Bolton et al., 1994; Bailey et al., 1995) and linkage analyses (Veenstra-VanderWeele and Cook, 2004) have confirmed the roles of a number of possible candidate genes, and the two

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different approaches mentioned above – the subgroup model and the severity model – seem both very promising, a clear etiological cause for ASD has not yet been demonstrated. Some researchers hypothesize that other neurobiological processes in addition to genetic processes, occurring at critical sensitive periods for brain development, might exert a pathogenic role. Indeed, the continuous gradient hypothesis fits well with a disease model where epigenetic factors – either stemming from the environment or originating from within the organism itself – could modulate a basic genetic vulnerability for autism, leading to a continuum of severity of symptoms. This view is consistent with the increasingly recognized role of epigenetic modulation ("plasticity") of the genome, resulting in steady modifications of phenotypes (Zhao et al., 2007) and with the so-called "triple helix model" of organism development (Lewontin, 2000), according to which the outcome of development depends on three different but interacting players: genetic mechanisms, the context of the organism and the environment.

Neuroanatomical and Neurofunctional Features of Autism Spectrum Disorders in Relationship to Brain Sexual Dimorphisms

At present, data on neuroanatomical alterations in ASD are still too scanty and not sufficiently robust to be convincingly linked to specific neurofunctional features in ASD. Brain regions showing more robust neuroanatomical alterations, in vivo or post-mortem, include the temporal cortex, the hippocampus, the amygdala and the cerebellum (reviewed in Courchesne et al., 2005; Keller and Persico, 2003; Palmen et al, 2004; Schumann and Amaral, 2006).

The amygdala and the hippocampal formation show sexually dimorphic patterns of development in humans, with amygdala volumes increasing significantly more in males than in females and hippocampal volume increasing more in females (Giedd et al., 1997). Observations in rodents are partially consistent with these human findings. The amygdala has been found to be significantly larger in male mice (Koshibu et al., 2004). In the hippocampus two interesting effects have been described: (1) the dentate gyrus of the hippocampus has been found to be larger in male rats (Roof, 1993) and also in some mouse strains but not in others (Tabibnia et al., 1999); (2) a lateralization of the volume of the dentate gyrus has been found, with the volume of the right dentate gyrus being larger than the left, this effect being observed in both sexes. Interestingly, testosterone has been implicated in both effects. Observations in mice are also consistent with a peculiar sensitivity of the hippocampus and amygdala to sex hormones, since these two structures have been found to be disproportionately larger in adult compared to peripubertal mice (Koshibu et al., 2004).

The existence of sexual dimorphisms in temporal cortex and cerebellum has not been sufficiently investigated to date, as the bulk of animal model research has focused on regions most directly related to reproductive behaviours. However,

recent work suggests the existence of robust sex differences in the expression of oestrogen receptor (ER) α, ERβ and the ER G-protein-coupled receptor 30 (GPR30) in the cerebellum and amygdala of newborn hamsters (Canonaco et al., 2008). It will be very important to confirm these observations as well as extend them to the human brain.

Theories Accounting for the Male Prevalence of ASD

The existence of a sex bias in ASD, with a male to female sex ratio of 3–4:1 for autism (Lord and Schopler, 1987) and 8–9:1 for AS (Wing, 1981), as well as some characteristics of cognitive functioning and emotion perception in this condition have led some researchers to link autism to "maleness". A very intriguing theory developed by Baron-Cohen et al. considers autism as an "extreme form of male brain" (Baron-Cohen and Hammer, 1997; Baron-Cohen, 2002, 2003). According to the *Extreme Male Brain (EMB) Theory*, in fact, autistic individuals show an extreme pattern of the typical male brain functions.

This theory moves its steps from the good deal of evidence for a sexual dimorphism both in neuropsychological features (Kimura, 1999) and in brain neurobiology (Kimura, 1999; Allen et al., 1989; Aboitiz et al., 1992).

Indeed women are reported to be better, on average, at verbal and social tasks, showing stronger ability in "empathy", while men, as a group, are superior at "systemizing", targeting and visuo-spatial tasks.

At such tests as the "Empathy Quotient" (EQ), "Reading the Mind in the Eyes" or "Facial Expressions", females score higher than males but autistic individuals score even lower than unaffected males (Baron-Cohen et al., 2001; Baron-Cohen and Wheelwright, 2004). On the other hand, an opposite personality profile has been shown for the "Systemizing Quotient" (SQ) with males scoring higher than females and people with ASD scoring highest of all (Baron-Cohen et al., 2003). The same pattern of results has been obtained on the "Embedded Figures" Task and "Mental Rotation" Task (Baron-Cohen, 1998; Jolliffe and Baron-Cohen, 1997; Ring et al., 1999).

Sex differences have also been found in the pattern of cerebral lateralization, with some studies reporting women to be less lateralized for some cognitive functions and language, as displayed, for example, by a more symmetric pattern at the dichotic listening test (McGlone, 1980; Hiscock et al., 1993). However, controversy exists as other studies have not replicated these findings or have found left-handedness to be more common in males (Sommer et al., 2008).

Hemispheric Lateralization, Brain Sexual Dimorphisms and Foetal Testosterone

Sex hormones exert important organizing effects, determining both the anatomical and the functional asymmetry of the brain, present from birth or arising during

childhood with different developmental trajectories (Giedd et al., 1996). Consistent with this view, an important study by Goldstein et al. (2001), carried out on normal adult brains, has reported greater sexual dimorphisms in the brain volume of those regions that express high levels of androgen and oestrogen receptors during foetal development.

Indeed it has been presumed that sexual differentiation of the nervous system is primarily established perinatally and then actively maintained throughout life. Furthermore, evidence has been provided in rats that the pubertal hormone surge contributes to the post-natal preservation of sexual dimorphisms through sex-specific modulation of new cell addition to sexually dimorphic brain regions (the anteroventral periventricular nucleus of the hypothalamus, the sexually dimorphic nucleus of the preoptic area, and the medial amygdala). For each region, the sex that gains more cells during puberty has a larger volume in adulthood. Removing gonadal hormones before puberty eliminated these sex differences, indicating that gonadal steroids direct the addition of new cells during puberty to maintain and enhance sexual dimorphisms in the adult brain (Ahmed et al., 2008).

Brain sexual dimorphisms as well as sex differences in the hemispheric lateralization represent interesting target areas to be linked with the core symptoms of ASD.

While both phenomena may be related, it is important to distinguish between hemispheric lateralization and brain sexual dimorphism Tabibnia et al. (1999) suggested that androgen receptors may play a role in development of laterality in the dentate gyrus of the hippocampus, independently of any sexual dimorphism in this structure. In their study, they examined mice with a defective structural gene for androgen receptors (testicular feminization mutant or tfm mice) on a C57/BL6J background and found that the right granule cell layer volume in the dentate gyrus of the hippocampus was greater than the left in the wild-type C57/BL6J mice in both sexes. Interestingly, the lateralization of the granule cell layer volume was lost in the tfm male mice or in the tfm-carrier females (partially androgen-insensitive), indicating that the lateralization effect in the dentate gyrus of the hippocampus was dependent on the action of androgens in both sexes.

With respect to hemispheric lateralization, some authors tried to relate the different pattern of cerebral functional lateralization to a different connectivity between the hemispheres as a consequence of different exposure to androgens, in particular testosterone, in the early development of the central nervous system in humans and non-human species (Reviewed in Wisniewski, 1998).

A number of animal studies have reported a link between early testosterone exposure and hemispheric specialization (Diamond, 1991; Westergaard et al., 2000).

The two main theories postulating a linkage between foetal testosterone (FT) and neuroanatomical and functional asymmetries in the brain and lateralization in humans are known as the “Callosal Hypothesis” (Witelson and Nowakowski, 1991) and the “Geschwind–Behan–Galaburda Hypothesis” (Geschwind and Galaburda, 1985a,b,c).

According to the "Callosal Hypothesis", increased hemispheric lateralization in males may result from the pruning of callosal axons – partially mediated by testosterone – during foetal and neonatal development. It is suggested, indeed, that increased levels of FT may be related to a greater axonal pruning and in turn to a decreased connectivity and a stronger right-hand preference in men. However, this effect was not observed in women and it was hypothesized that different mechanisms might apply to each sex (Witelson, 1985, 1989). Further support to this theory comes from studies of premature babies showing that babies with very low birth weight (<1000 g) and high prematurity (weeks 26–29) – two factors which may have interfered with normal callosal axonal pruning – were found to be more left-handed (O'Callaghan et al., 1987). Also some authors, using samples of amniotic fluid from normal female foetuses at 16 weeks of gestation, found a correlation between higher FT levels and strong right-handedness and left-hemisphere lateralization for speech at age 10 arguing in favour of the Callosal Hypothesis (Grimshaw, 1995). This theory was also supported by evidence, in post-mortem studies, of a smaller corpus callosum in right-handed men than non-right-handers (Witelson, 1985, 1989). However, these findings have been challenged by other studies. It should also be noted that cross-sectional studies are negatively affected by the enormous variability of the size of the corpus callosum (Giedd et al., 1999). Kertesz et al., based on magnetic resonance imaging, argued with the notion of increased callosal connectivity in left-handers, women or individuals with less-lateralized function, finding that callosal areas did not correlate with brain size or with measures of lateralization for hand performance, dichotic listening or visual field preference (Kertesz et al., 1987). Also recent studies using diffusion-tensor imaging (DTI) and high-resolution morphological MRI revealed a larger total callosal area in right-handed subjects as compared to left-handed subjects and in males as compared to females and an increased anisotropy and diminished diffusion in left-handers, completely contradicting Witelson's proposal (Westerhausen et al., 2004).

With respect to the role of FT in brain lateralization, the second main hypothesis, formulated by Geschwind–Behan–Galaburda (GBG), inferred that FT might cause a slowing in the development of the left hemisphere with a consequent compensatory growth in the right hemisphere, creating a reverse organisation of the cerebral lateralization. That is, left- and right-handedness might be associated with high and low FT levels, respectively. Also a corollary of the GBG model claimed that FT might influence the development of the immune system too, accounting for the associations between immune functioning and laterality (Geschwind and Galaburda, 1985a,b,c).

Although very promising especially for the emphasis on the intra-uterine environment, the GBG hypothesis has also been refuted as subsequent studies failed to support its predictions (Bryden et al., 1994; Obrzut, 1994; Berenbaum and Denburg, 1995). In particular, a study by Gilmore et al. (2007) found the left hemisphere to be *larger* than the right in male neonates in comparison to female neonates, which is exactly the opposite of what would be predicted by the GBG hypothesis.

Foetal Testosterone and Autism

Turning our focus on the neurobiological link between autism and maleness in the light of these two theories – that although criticized, have highlighted the importance of prenatal environmental factors such as male sex hormones on cognitive and psychological brain development – some authors tried to propose a candidate mechanism for the association between maleness and autism just in the role of FT in early brain development (Baron-Cohen et al., 2004).

The "Extreme Male Brain" theory, in fact, argues that prenatal testosterone exposure is a strong candidate for contributing to sexual dimorphism in human behaviour, including social development, and may represent a risk factor for conditions characterized by social impairments, particularly autism spectrum conditions (reviewed in Knickmeyer and Baron-Cohen, 2006).

Indeed elevated FT levels, measured in amniotic fluid, have been positively correlated with a number of autistic traits and inversely correlated with social development and empathy (Knickmeyer et al., 2005, 2006). Indirect evidence in support of this theory comes from a large body of research on the neural and behavioural effects of early exposure to testosterone and its metabolic derivatives both in animal models and genetic disorders causing increased virilization during gestation (Hines, 2006, review).

Hormonal manipulation during critical periods of early life leads to largely consistent outcomes across animal species. Indeed females of both rodents and non-human primates exposed respectively neonatally and prenatally to androgens showed neural and behavioural masculinization (Goy and McEwen, 1980).

Furthermore, studies of individuals with genetically determined prenatal endocrine conditions, such as women with congenital adrenal hyperplasia (CAH) or men with complete androgen insensitivity syndrome (CAIS), provided extensive information on the consequences of prenatal androgen abnormalities.

The prenatal exposure to unusually high levels of testosterone in girls with CAH, a condition characterized by a deficiency of cortisol biosynthesis leading to compensatory increase of ACTH secretion and a shift toward androgens, seems to be linked to increased male typical play behaviours, including increased preferences for toys and activities usually chosen by boys and for male playmates, despite parents encouragement for sex-appropriate behaviours (Hines, 2004; Pasterski et al., 2005). Furthermore, increased autistic traits, as shown by higher scores on Autism Spectrum Quotient (AQ), have been reported in women with CAH, suggesting that prenatal exposure to high levels of testosterone may be involved in vulnerability to autism (Knickmeyer et al., 2006a).

The opposite pattern, with a female-typical psychosexual development, has been shown by men with complete androgen insensitivity syndrome (CAIS), an X-linked disorder characterized by a total lack of functional androgen receptors (Hines et al., 2003; 53; Wisniewski et al., 2000).

Other researchers have looked more directly at the possible link between autism and prenatal and current testosterone. There is evidence that the ratio of the lengths of the second and fourth digit (2D:4D) may be negatively correlated with prenatal

testosterone and smaller 2D:4D ratio has been considered as a "male finger pattern". Some studies found that the 2D:4D ratio of children with autism, their siblings, fathers and mothers were lower than population normative values and suggested that 2D:4D ratio might be a possible marker for autism which could implicate prenatal testosterone in its aetiology (Manning et al., 2001). Although very intriguing, the findings from 2D:4D ratios have not been always consistent, and the results may vary according to the age, race, what is measured, the hand selected and the method used (Manning et al., 2005, 2007; Robertson et al., 2008).

Finally, signs of precocious puberty have been reported in ASD subjects (Tordjman and Ferrari, 1992) and elevated rates of testosterone-related disorders such as polycystic ovary syndrome, irregular menstrual cycle, dysmenorrhoea, hirsutism, severe acne, epilepsy, tomboyism, and bisexuality or asexuality have been found in women with autism spectrum conditions (Ingudomnukul et al., 2007), suggesting post-natal hormone abnormalities in testosterone production or sensitivity, though the relationship between FT and post-natal testosterone levels has not been clarified yet.

Assessment of the effect of testosterone on brain development in humans (and other mammals) is complicated by the fact that there are two different testosterone peaks in males, the first occurring during early gestation, coincident with the period when male testes start to secrete the hormone, the second occurring around birth. Both peaks could have important organizing effects on the male brain.

Adding complexity to the issue, although it is widely held from animal models that testosterone exerts its masculinizing effect on the brain following aromatization into estradiol (as discussed below), recent experiments involving the administration of the non-aromatizable androgen dihydrotestosterone (DHT) to newborn female mice showed that perinatal testosterone exerts masculinizing effects also via androgen receptors (Bodo and Rissman, 2008). In conclusion, clarifying the role of testosterone for brain development appears to be a key goal for autism research and will probably require a large experimental effort in the years to come.

The Role of Oestrogens in Sexually Dimorphic Anatomy and Behaviour

After having considered the role of FT on sexually dimorphic traits and brain lateralization, we now turn our attention to oestrogens.

While sexually dimorphic expression of oestrogen receptors has been consistently reported in areas related to reproductive behaviour, such as the hypothalamus, evidence of sexually dimorphic oestrogen pathways in areas that are not directly related to reproductive behaviour is much more limited. Recent work by Canonaco et al. (2008) suggests the existence of robust sex differences in the expression of oestrogen receptor (ER) α, ERβ and the ER G-protein-coupled receptor 30 (GPR30) in the cerebellum and amygdala of newborn hamsters.

It is important to remember that brain androgen and oestrogen pathways are coupled together, because androgen precursors (androstenedione and testosterone) are converted into oestrogens (oestrone and estradiol respectively) in the brain, by

the cytochrome P450 aromatase enzyme. Thus, it seems, from rodent models, that estradiol, not testosterone, is the true "masculinizing hormone", at least for some brain regions. Many neurons express P450 aromatase, in particular Purkinje cells (Ukena et al., 1998). Indeed, according to this hypothesis, because of the perinatal testosterone surge, the developing male brain is exposed to high levels of oestrogens derived from neural aromatization of testosterone, while the female brain is exposed to high oestrogen levels only after puberty.

The aromatization hypothesis further implies that female rodents might need to be protected prenatally from the effects of the oestrogens produced by the placenta or foetal gonads. It has been interestingly proposed that alpha-fetoprotein (AFP) may play a role in brain sexual differentiation by binding with high-affinity oestrogens produced by the placenta in female rodents. This hypothesis has been tested in the AFP mutant mouse ($AFP^{-/-}$), showing a clear pattern of masculinization and defeminization in the brain and the behaviour of female $AFP^{-/-}$ mice (Bakker et al., 2006).

In agreement with this view, Bakker and Baum suggested that the defeminizing effect of prenatal estradiol in male rodents was avoided in foetal females by a protective role of AFP whereas oestrogens exerted their feminizing action postnatally, as expected, in genetic females (Bakker and Baum, 2008).

However, it should be noted that human AFP does not seem to bind oestrogens although it demonstrated an antioestrogenic activity in oestrogen-sensitive breast cancer (Bennett et al., 2002) and that androgens seem to be the main hormones causing brain masculinization in primate species (Wallen, 2005).

Turning back to the role of oestrogens in sexually dimorphic neuroanatomy, one of the regions examined in neural sexual differentiation studies, the sexually dimorphic nucleus of the medial preoptic area (SDN-POA) – which is involved in many behaviours including masculine sexual and social activities – was found to be five to six times larger in volume in male than in female rats (Gorski et al., 1978). Furthermore it has been found that brief exposure of newborn female rats to very high levels of estradiol masculinizes the volume of the SDN-POA by reducing apoptotic cell death (Arai et al., 1996).

Foetuses and pups seem also extremely sensitive to even low oestrogen doses. For example, it has been found that pregnant mice with deletion of the 5α-reductase gene – exposed for this reason to increased levels of testosterone and, via aromatization, to high estradiol levels during gestation – were characterized by a strongly reduced litter size and foetal loss. Furthermore, administration of an oestrogen receptor antagonist or inhibition of aromatase reversed the high rate of foetal death in the mutant mice, and estradiol treatment of wild-type pregnant mice caused foetal wastage. Taken together, these findings indicated possible oestrogen toxicity during pregnancy (Mahendroo et al., 1997).

In summary, it might be that oestrogen, rather than testosterone, is the critical hormone to understand the skewed sex ratio in autism.

Several studies have begun to dissect out the mechanisms by which oestrogen pathways affect adult behaviour. Oestrogens are well known to exert their effects through two different oestrogen receptors (ER), ERα and ERβ. ERα is thought to be the fundamental receptor-mediating oestrogen action on reproductive organs

and reproductive behaviour, while ERβ is thought to mediate at least some of the effects of oestrogens on behaviours that are not specifically associated with reproduction, such as locomotor activity, arousal, fear responses, anxiety and learning (Krezel et al., 2001). In the same study, ERβ knockout (KO) mice displayed disrupted GABAergic function in the medial amygdala. ERβ appears to be the principal oestrogen receptor expressed in brain areas like the cerebral cortex, the hippocampus and the cerebellum (Bodo and Rissman, 2006). ERα aαnd ERβ have also different functions during brain development, as suggested by the fact that ERβαKO mice show defects of neuronal migration while ERαKOs do not (Wang et al., 2003). Interestingly, a recent genetic study has revealed significant association of the aromatase gene with Asperger syndrome, and the ERβ gene with the Autism Spectrum Quotient (AQ) and the Empathy Quotient (EQ) (Chakrabarti et al., 2009), further underscoring the potential involvement of oestrogen pathways in ASD.

More recently, work by Choleris et al. (2003, 2006) in mice has added a new perspective on the behavioral consequences of developmentally altered oestrogen pathways, proposing the hypothesis that a "gene micronet" made up of ERα and ERβ, oxytocin (OT) and its receptor (OTR) may control development of social cognition. According to this hypothesis, oestrogens act on the oxytocin system in the hypothalamus and in the limbic forebrain, particularly in the amygdala, as suggested by the fact that ERαKO and ERβKO, as well as OTKO female mice, were found to be impaired in social recognition (Choleris et al., 2003, 2006).

Indeed, previous studies on animal models have demonstrated the critical role of OT and the related peptide vasopressin (VP) in stress, coping responses and social, adaptive behaviours influencing social affiliation and social recognition, sexual and maternal behaviours and anxiety and reactivity to stressors (Carter and Keverne, 2002; Pedersen and Boccia, 2006).

Direct effects on human behaviours by OT have also been reported in past researches. In particular OT seems to increase trust in social interaction (Kosfeld et al., 2005) and to reduce responses to social stress (Heinrichs et al., 2003) and intranasal administration of OT improved the ability to infer the mental state of others from social cues of the eye region (Domes et al., 2007), encouraging researchers to explore whether the so-called trust hormone could represent a potential autism treatment (Opar, 2008).

Furthermore, clinical studies on ASD reported lower peripheral levels of OT (Modahl et al., 1998) and higher levels of a precursor form may be less active of OT (OT-X) (Green et al., 2001). Also patients with autism spectrum disorders showed a significant reduction in repetitive behaviours following oxytocin infusion in comparison to placebo infusion (Hollander et al., 2003) and intravenous oxytocin administration has been shown to facilitate retention of social information in those with autism (Hollander et al., 2007). Additional evidence for a link between OT and ASD comes from genetic studies, which associated the gene for the OT receptor (OTR) with autism (Auranen et al., 2002; Shao et al., 2002; Wu et al., 2005).

Noteworthy is that OT and VP expression seems sexually dimorphic in some cases, as cited in the Carter review (2007), with some studies reporting higher OT levels in females versus males and on the contrary enhanced VP function in males as compared to females. Also it seems that intranasal VP administration differentially

affects social communication in the two sexes, promoting agonistic responses in men and affiliative responses in women (Thompson et al., 2006) and that VP exerts a more crucial role in males than females as pointed out by the social impairment displayed in male but not female V1aR-deficient mice (Bielsky et al., 2004, 2005).

Also the functional interactions among OT, reelin and GABA could exert a pivot role to the features of ASD, such that reelin is secreted by specific subtypes of GABAergic interneurons in the adult cortex and hippocampus and a significant reduction in OTR has been observed in heterozygous *reeler* mice (Carter, 2007). Indeed, epigenetic methylation of the reelin or OTR promoter regions induced by developmental events might cause gene silencing and consequent down-regulation of the protein expression.

Oestrogens, Reelin and Cerebellar Circuits

The *Reelin* gene has been implicated in the aetiology of neurodevelopmental disorders such as schizophrenia and autism (Abrahams & Geschwind, 2008; Fatemi, 2005; Keller & Persico, 2003). Interestingly, a common variant of the *Reelin* gene has been found to increase schizophrenia risk only in women (Shifman et al., 2008), confirming that gene–sex interactions can be important for neurodevelopmental disorders.

We have recently begun to assess the interaction between reelin and sex hormones through topical administration of estradiol into the cisterna magna over the cerebellum in newborn mouse pups (Biamonte et al., 2009). Male heterozygous *reeler* mice (*rl*/+) show a decreased number of Purkinje cells (PC) in the cerebellum at post-natal day 15 (P15), while female *rl*/+ mice do not show any PC loss. This represents an interesting example of gender-dependent modulation of phenotypic expression of a mutation in the hemizygous state, whereby males are affected while females are not. What is the reason for this sex-dependent phenotypic expression of the *rl* allele? Starting from the hypothesis that this phenomenon could be mediated by differential exposure to androgen/oestrogens during foetal or early post-natal development, we have shown that pharmacological treatments with the oestrogen receptor agonist 17-β-estradiol (17β2) at P5 into the cisterna magna of mice increase PC numbers in male *rl*/+ but have no effect in female *rl*/+ or male/female wild-type (wt) mice. Conversely, treatments with the oestrogen receptor antagonists tamoxifen or ICI 182780 decreased PC number in wild-type and *rl*/+ females, but did not affect male's PC numbers (Biamonte et al., 2009). Interestingly, in Reelin-null mice (*rl/rl*) PC loss is not affected by gender nor are PC numbers changed by 17-β-estradiol. RT-PCR analysis indicated that heterozygosity leads to a 50% reduction of reelin mRNA in the cerebellum in both sexes, and that 17β-E up-regulates reelin mRNA, particularly in rl/+ males; reelin mRNA upregulation is associated with an increase of all major reelin isoforms. These results, together with data on the levels of androgens and oestrogens in the mouse cerebellum during early post-natal development, suggest that the local androgen/oestrogen ratio can modulate the phenotypic expression of the Reelin mutation in the heterozygous state. In humans, reelin alleles associated with low levels of gene expression may interact with

variable perinatal levels of neuroactive steroids, leading to gender-dependent differences in genetic vulnerability.

Furthermore longitudinal behavioural analysis in these mice evidenced a genotype-by-estradiol interaction for number of ultrasonic vocalizations (USV) in response to separation from the mother at P7. Basal levels of USV in *rl*/+ females were significantly lower than in wild-type females. Estradiol reverted this profile. Also in the homing test on P9, a significant lower percentage of *rl*/+ mice reached the nest area than corresponding wild-type pups. In the absence of motor changes, this indicates a genotype-dependent alteration in sensitivity to or in central processing of social stimuli. Remarkably, also this deficient profile was reverted by neonatal estradiol treatment. When adult male mice were assessed in an attentional set-shifting task, involving the formation of new rules to obtain a palatable reward, *rl*/+ subjects showed a higher number of perseverative responses. Neonatal estradiol abolished the differences between *rl*/+ and wild-type mice (Laviola et al., 2008).

Taken together, these anatomical and behavioural observations support the male *rl*/+ mouse as a model to assess the complex interactions between genetic risk, altered sex hormone levels and a specific neural circuit (the fronto-cerebellar loop) that is relevant for ASD.

Concluding Remarks: Making Sense of the Complexity

Some of the most prominent biological candidates involved in autism – interacting between each other and influenced by sex steroids – include, among others, neuropeptides such as oxytocin and arginine–vasopressin, neurotransmitters like serotonin and GABA, and neurosecretory proteins such as reelin. Testosterone and related steroids and in particular the estradiol/testosterone ratio may play an important role through their receptors in several hormone-sensitive neural regions interacting with many biological factors and influencing patterns of programmed cell death, neurochemical processes, and neural connectivity between brain regions. The cerebellum and the amygdala are emerging as two brain regions where the interplay between genetic risk factors and neurosteroids could turn out to be crucial for autism. In autism research, there is presently a strong need for robust experimental models that should be taken into account the complex interactions between genes, specific neural circuits and the environment.

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Neuroanatomical-Behavioral Correlates in Autism: A Working Hypothesis

Ricardo M. Vela

Introduction

A considerable amount of scientific knowledge has accumulated, since Kanner (1943) described the syndrome of autism 65 years ago. His description of this condition remains a classic contribution to psychiatric taxonomy and has profoundly influenced the work of several generations of clinicians and investigators (Volkmar et al., 1996). Unfortunately, Kanner's interpretation of parental psychopathology and disturbed parent–child interaction, and Bettelheim's further characterization of "refrigerator mothers" (Bettelheim, 1967) not only led to uncalled for blame and guilt of caretakers but misled early investigators about the etiology of autism. Neuroanatomical abnormalities in the brains of autistic individuals provide hard evidence of the neurobiological substrate of this disorder and relieve parents of the guilt of having inflicted, through bad parenting, this family-stressing psychopathology.

Autism is a heterogeneous, complex disorder associated with different etiological underpinnings. Our knowledge of the neural networks that guide and control complex social behaviors is incomplete (Bachevalier and Loveland, 2006). Although many gaps exist in the link between specific neuropathological abnormalities and behavioral manifestations in autism, this chapter attempts to formulate a working hypothesis to explain observed behavior as a manifestation of disturbed neuroanatomy. The focus of the chapter is on emotions and attachment behavior. This chapter does not attempt to present a comprehensive theoretical model. Due to our incomplete knowledge at the present time, this model is necessarily over-simplified. Nevertheless, it is hoped that this discussion will stimulate thinking about the perplexing behaviors, emotions, and social relatedness of individuals with autism, enrich the conceptualization of this syndrome, and suggest research experiments to prove or disprove the hypothesis and/or help interpret research results.

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Neuroanatomical Abnormalities in Autism

Early attempts to associate neuropathological findings to autism were confounded by comorbid neuropathology (Darby, 1976). In 1985 Bauman and Kemper reported a systematic neuropathological postmortem study using whole brain serial sections, of a 29-year-old man with autism, compared to a matched control (Bauman and Kemper, 1985). Subsequently five additional cases were studied, aged 9, 10, 12, 22, and 28 years old, mostly males (Bauman and Kemper, 1994, 2005). The results showed no gross abnormalities, but microscopic examination revealed mild cytoarchitectonic abnormalities in autistic brains compared to controls. Abnormal areas included the hippocampus, subiculum, entorhinal cortex, amygdala, mamillary body, septum, and anterior cingulate cortex. These areas showed reduced neuronal cell size and increased neuronal cell-packing density. No differences were found in the thalamus, hypothalamus, or basal ganglia, compared to controls. The abnormal areas comprise a major portion of the limbic system of the forebrain, closely connected by interrelated circuits. These limbic areas, which are abnormal in autistic individuals, are part of a circuitry important for memory, learning, emotion, and behavior (Bauman and Kemper, 2005; Papez, 1937).

Pyramidal neurons in the hippocampal areas CA1 and CA4 show decreased complexity and extent of the dendritic arbors in autism (Raymond et al., 1996). In the amygdala, the medial, cortical, and central nuclei consistently showed the most increase in cell-packing density. Abnormalities in the septal region were found in the vertical limb of the nucleus of the diagonal band of Broca (NDB) and the medial septal nucleus (MSN). Again cell-packing density was significantly and consistently increased in the MSN, as in the other abnormal structures. However, there was an age difference regarding cell size in the NDB. In the brains of the younger – 9-, 10-, and 12-year-old children – neurons were unusually large and adequate in numbers. By contrast, in the brains of autistic adults – 22, 28, and 29 years – neurons were small in size and markedly reduced in number (Bauman and Kemper, 1994, 2005). In a review of neuropathological findings in autism, Palmen and collaborators found 9 of 14 studied cases showed increased cell-packing density and smaller neuronal size in the limbic system (Palmen et al., 2004).

In another postmortem study, Schumann and Amaral (2006) studied 9 male autistic brains and 10 matched male controls, 10–44 years of age, using systematic stereological techniques. The method used was independent of volume measurement and thus unaffected by tissue shrinkage. Autistic individuals with comorbid seizure disorder were excluded from the sample, as epilepsy alone can affect the structure of the amygdala. There were no group differences in the volume of the amygdala or its subdivisions. However, there were significantly fewer neurons in the total amygdala of autistic subjects compared to controls, especially in the lateral nucleus. In a previous magnetic resonance imaging (MRI) study Schumann and collaborators (2004) looked at the neuropathology of the amygdala and hippocampus of children and adolescents with autism with or without mental retardation (MR), Asperger's syndrome, and controls. Children with autism, 7.5–12.5 years of age, had larger right and left amygdala volumes compared to controls, regardless of the

presence or absence of MR. Adolescent subjects, 12.75–18.5 years old, showed no differences in right or left amygdala volume or total cerebral volume between clinical subjects and controls. The findings indicate that the increase in amygdala volume in younger children is not due to MR. In children with autism the amygdala reaches adult size before adolescence (around 8 years of age) (Schumann et al., 2004; Schumann and Amaral, 2006) while normally developing children and adolescents maintain progressive amygdalar growth through adolescence. Although the amygdala is of equal size in autistic adolescents compared to controls, there are basic characteristics of its neuroanatomical or functional organization that are different in autistic subjects.

Aside from the abnormalities found in the forebrain limbic system, the only other abnormal areas reported by Bauman and Kemper were in the cerebellum and related inferior olive. Throughout both cerebellar hemispheres, there were a decreased number of Purkinje cells and a variable decrease in granule cells. The areas of most marked cell decrease were the posterolateral neocerebellar cortex and the adjacent archicerebellar cortex. In addition there were abnormalities found in the fastigial, emboliform, and globose nuclei of the cerebellum. Again these changes showed a dichotomy between younger and older subjects. In children, neurons in these nuclei, as well as the dentate nucleus, were enlarged in size but adequate in number. By contrast, in the cerebellar nuclei of adult subjects, neurons were small and pale and decreased in number, compared to controls. Palmen et al.'s (2004) review of neuropathological findings in autism found 21 of 29 studied cases showed a decreased number of Purkinje cells. A more recent study (Whitney et al., 2008), using modern stereology and a more reliable (immunostain) marker of Purkinje cells, showed no significant difference in the density of Purkinje cells between six autistic brains and four controls. However, three of the six autistic brains had significantly reduced Purkinje cell numbers, compared to control brains. Finally, olivary neurons, which are closely related to the cerebellar cortex, also showed age-related abnormalities (Bauman and Kemper, 1994, 2005). Children had neurons that appeared normal but were enlarged, while adult brains had adequate number of olivary neurons, but these appeared small and pale.

Limbic and Cerebellar Abnormalities and their Functional Relationship with Autistic Symptoms

The following section explores the five areas most consistently found to be abnormal in postmortem studies of autistic brains – amygdala, septal nucleus, hippocampus, anterior cingulate cortex, and cerebellum – and discusses their hypothetical relationship to emotional and interpersonal psychopathology in autism. Note that other brain areas, which are inconsistently abnormal in neuropathological studies, may express inconsistent associated symptoms, variations in psychopathological subtypes, or inconsequential anomalies and are not discussed in this section.

Amygdala

In 1888 Sanger Brown and Edward Albert Schäfer published the results of surgical ablations of parts of the brain of monkeys and correlated these with behavioral observations and interpretations. Two of the animals received extensive lesions of the temporal lobe. The authors reported that these monkeys, who had been previously fierce, became tame postoperatively. They no longer seemed to understand the meaning of sounds and sights. Every object seemed to appear strange and had to be mouthed, tasted, and smelled repeatedly (Brown and Schäfer, 1888). These observations were virtually forgotten by the scientific community for half a century until Heinrich Klüver and Paul Bucy wrote a series of reports in which they described the unusual effects of bilateral temporal lobectomies in rhesus monkeys (Klüver and Bucy, 1937, 1939). In contrast to Brown and Schäfer, who were not able to make sense of their observations, Klüver and Bucy benefited from the ground-breaking paper by Papez (1937) which had broken away from the long-held assertion that the area classified as the rhinencephalon or "nose brain" was only related to olfaction, but belonged to a circuit involved in emotional expression. These monkeys showed no fear or aggression, approached animate or inanimate objects without fear and had a compulsive tendency to examine every visible object or investigate it with their mouths. Subsequently, experiments showed amygdala lesions had a direct effect on the animals' social status in cages (Rosvold et al., 1954) or in the wild (Dicks et al., 1968).

Animals and humans with Klüver–Bucy syndrome or with amygdala or amygdala-area lesions or ablations have been proposed as being models of autism or to express behavior seen in people with autism (Baron-Cohen et al., 2000; Bachevalier, 1991, 1994, 2005). Prather and collaborators (2001) used ibotenic acid lesions, to specifically destroy the amygdala's cell bodies, while sparing adjacent structures and fibers of passage. Macaque infant monkeys lesioned at 2 weeks of age, and quickly returned to their mothers, displayed less fear of objects, but more fear than controls in dyadic (peer) social interactions at 6–8 months of age. There were no differences in mother–infant interactions between amygdala-lesioned and control infants. Moreover, these investigators did not find the lesions resulted in autistic-like behaviors.

These findings by Prather's group are difficult to interpret and raise several important considerations. Lesion studies may shed light into the functions of the amygdala, but they are not to be conceptualized as equivalent to microanatomic abnormalities found in autistic brains, the significance of which we do not fully understand with our current state of knowledge. Moreover, the amygdala is a highly plastic brain region (LeDoux, 2000; Schumann et al., 2004; Schumann and Amaral, 2006). If the amygdala is surgically ablated, especially in infancy, other structures may take over functions necessary for survival. This, however, may not be the case for abnormally functioning neurons and structures which most likely have compromised plasticity. Abnormalities in microstructure may reflect non-plastic, malfunctioning, neuronal abnormalities, which, in turn, may have emotional, behavioral, and psychopathological manifestations.

Kanner's seminal publication describing 11 autistic children used the word "autistic" to describe a remarkable lack of interest in other people and felt that this was a central defining feature of the syndrome (Kanner, 1943; Fein et al., 1986; Volkmar et al., 1996). All autistic children fail to show the usual relatedness infants show to their parents and caretakers. They frequently lack the "social smile" as infants. Many do not recognize or discriminate most important people close to them, like parents and siblings.

The formation and maintenance of social relationships is a complex process. Lim and Young (2006) have proposed a simplified conceptual framework of four levels of attachment. The first level, *social recognition,* requires the individual's ability to recognize and remember other individuals before forming social relationships. In humans, social recognition is primarily visual. Lesions in the fusiform gyrus can abolish the ability to recognize faces. Neuroimaging studies of autistic patients viewing human faces show decreased amygdala and fusiform gyrus activation compared to controls (Critchley et al., 2000). Others (Kleinhans et al., 2008) have found no differences in the activation of the lateral fusiform gyrus (fusiform face area, FFA) between autistic subjects and controls. Rather, autistic subjects showed decreased *connectivity* of the FFA to the left amygdala and posterior cingulate cortex. In this study, greater social impairment was associated with reduced FFA-amygdala connectivity (Kleinhans et al., 2008). Autistic individuals also failed to show amygdala activation when required to interpret emotions based on the perception of another person's eye expression (Baron-Cohen et al., 2000). This lack of activation of the amygdala (and its network connections) may be present from the neonatal period and may explain, at least in part, the reduced or abnormal eye contact seen in autistic infants and their lack of interest in the human face.

The amygdala serves as the seat of social and emotional intelligence (Joseph, 1996), and autistic individuals are particularly deficient in this area. The amygdala monitors and abstracts motivational significance from an array of multimodal sensory stimuli and has the ability to discern and express even subtle social-emotional nuances. It analyzes information and transfers it back to the neocortex for further processing. It is the main limbic area that attaches emotional significance to sensory input (Devinsky and D'Esposito, 2004). Autistic individuals – even as adults – have extreme difficulties in understanding the nuances of social cues (Volkmar et al., 1996). This seems to be a major part of the reason they have difficulty making friends, behaving appropriately in social situations and responding empathically to others. When parents attempt to hold them, autistic children may fail to conform to their parent's posture or may actively resist being held (Fein et al., 1986; Volkmar et al., 1996). This may further impinge upon a vicious cycle, for in order for the amygdala to develop normally, an infant needs to receive tactile stimulation. If infants are exposed to neglectful environments or if adequate stimulation is not provided, developing neurons will establish/maintain abnormal interconnections or whither and die at an accelerated rate (Joseph, 1996, 1999). Harlow's monkeys raised with surrogate terry cloth mothers developed extreme behaviors including staring into space, stereotypical behaviors, rocking for long periods of

time, compulsive habits, and self-injurious behavior (Harlow, 1992; Harlow and Harlow, 1965).

Autistic individuals appear to be emotionally non-expressive. Bryson, (2005) theorizes that lack of *expressed* emotion does not necessarily imply lack of *felt* emotion. She argues that people with autism are capable of experiencing a range of emotions, but the expression of these may not be readily apparent, as they tend to be expressed atypically. Bryson states that many people with autism seem only to be capable of differentiating between "good" and "bad" feelings. Internal physical sensations associated with emotions can be expressed as intense and overwhelming and may result in a state of overarousal. Freezing has been observed by Bryson in response to an emotional event.

The hypothalamus is phylogenetically a very ancient structure and is almost fully functional in human infants at the time of birth (Joseph, 1996). It appears to be normal in postmortem examinations of autistic individuals (Bauman and Kemper, 1994, 2005). Emotional states generated by the hypothalamus are primitive, diffuse, undirected, reflexive, and unrefined (Joseph, 1996). Stimulation of different parts of the hypothalamus may result in experiences of pleasure vs. displeasure, reward vs. aversion, or tranquility vs. raw emotionality. Longer lasting emotions require the recruitment of and interaction with other limbic nuclei, giving complex, higher order emotional reactions. These nuclei (e.g., the amygdala, septal nuclei) and the associated anterior cingulate cortex may not be properly or effectively recruited to control the stimulation of the hypothalamus of autistic individuals. Freezing responses, on the other hand, involve the stimulation of the periaqueductal gray matter (PAG), a downstream structure in the hierarchy of emotional control subservient to the actions of the hypothalamus (Panksepp, 1998).

Bryson's hypothesis further states that hypersensitivity to sensory inputs results in states of hyperarousal in autistic individuals. Sensory stimulation is experienced as unusually intense and information uptake is incomplete or distorted. This results in the adoption of an overly narrow beam of attention or development of "tunnel vision." Information is stored in separate, rather than conceptually or semantically related "folders" (Bryson, 2005). In view of the findings of neuronal abnormalities in autistic individuals' brains (Bauman and Kemper, 1985, 1994, 2005; Schumann and Amaral, 2006), one may further elaborate this hypothesis. Autistic individuals may be overwhelmed with sensory inputs because their amygdalas are unable to process information efficiently and accurately and transmit this processed information to other parts of the brain. As a result, the autistic individual may subjectively experience this as sensory overload. Processing of information by amygdaloid nuclei with abnormal neurons may affect the output and storage of information to other parts of the brain. "Tunnel vision" may be a compensatory, adaptive mechanism to cope with sensory overload. Storage of information in fragmented "folders" may be the result of abnormal (basolateral) neuronal processing and abnormal (centromedial) output to different areas of the brain.

Amaral and his group (Amaral, 2002) have proposed a working hypothesis that postulates that the amygdala is a protection device, designed to detect and avoid danger. It evaluates objects or organisms prior to interacting with them, and,

based on the outcome, coordinates a species-specific response through connections to other parts of the brain (neocortex, hippocampal formation, and subcortical areas). The amygdala in normal individuals reaches an adult size in late adolescence, but in autistic boys it is at adult size by 8 years of age and does not enlarge any further (Schumann et al., 2004, Schumann and Amaral, 2006). Amaral's group hypothesizes a biological defect in autism leads to a larger and more active amygdala, producing increased fear and anxiety and a higher stress response (Schumann and Amaral, 2006). Anxiety is a comorbid feature of autism (Muris et al., 1998). Abnormal processing of fear during development may contribute to the behavioral symptoms seen in autism (Schumann et al., 2004).

Septal Nuclei

As stated previously, abnormalities in the septal region have been consistently found (Bauman and Kemper 1985, 1994, 2005). The septal nuclei maintain a counterbalancing relationship with the amygdala, exerting inhibitory influences on the amygdala, while, in turn, the amygdala acts to facilitate or inhibit septal functioning. In conjunction with the medial hypothalamus, the septal nucleus appears to exert quieting and dampening influences on arousal, which, in turn, facilitates the maintenance of selective attention and memory. The septal nuclei also maintain antagonistic influences on the hypothalamus, facilitating the actions of the medial hypothalamus, thereby reducing extremes in emotionality and arousal, and maintaining a state of quiescence and readiness (Joseph, 1996).

Electrical stimulation of the septal nuclei inhibits aggressive behavior. If the septal nuclei are destroyed, limbic counterbalances are removed, resulting in increased aggressive behavior. The rage and irritability are soon replaced by indiscriminate socializing and extreme need for social/emotional contact. According to Joseph (1992) there appears to be a counterbalancing relationship between the septal nuclei and the amygdala. After the initial aggressive behavior subsides, patients suffering from septal nuclei lesions show indiscriminate socializing and need for social and physical contact. According to Joseph, this behavior appears to be the result of an unopposed amygdala, unrestrained by the inhibitory influences of the septal nuclei. This suggests that the amygdala (which is abnormal in autism) promotes indiscriminate social behavior, while the septal nuclei (which is also abnormal in autism) seems to counteract generalized socializing tendencies, and instead (together with the anterior cingulate gyrus) act to promote selective attachments. Specific and long-term attachments, according to this hypothesis, require the maturation and involvement of the septal nuclei and cingulate gyrus (Joseph, 1992, 1996).

All autistic children fail to show the usual relatedness normal children demonstrate to parents and other people. They show deficient attachment behavior and early failure of person-specific bonding. They do not seem to recognize or differentiate the most important people in their lives, such as parents, siblings, and teachers. In infancy, they do not show signs of separation anxiety when facing strangers. All these deficiencies in the attachment behavior of autistic individuals may be related to

complex abnormal interactions between the amygdala, septal nuclei, and the closely, functionally related, anterior cingulate gyrus.

Anterior Cingulate Cortex

The anterior cingulate cortex (ACC) is the largest limbic structure and forms the upper layer of the emotional brain, attending to evaluating and reacting to novel and complex behavior and monitoring behavioral output (Devinsky and D'Esposito, 2004). The affect division of the ACC (Brodmann's area 25, 33 and rostral area 24) has rich interconnections with the septal nuclei, amygdala, hypothalamus, mamillary bodies, hippocampus, dorsomedial nucleus of the thalamus, and periacqueductal gray (PAG) (Devinsky and D'Esposito, 2004; Joseph, 1996). The ACC is involved in processing and modulating expression of emotional nuances, with which autistic individuals have considerable difficulties. The ACC is paramount in the formation of long-term attachments and maternal behavior. It is capable of producing emotional sounds and the separation cry (Joseph, 1996; MacLean, 1985). Autistic children appear happiest when left alone. They do not usually turn to others to express their feelings, seek physical comfort, or respond to other individual's expression of feelings. Moreover, autistic children may fail to discriminate between different adults and tend not to follow parents around or notice their homecoming (Fein et al., 1986).

Primates with cingulate destruction will cease to groom or show acts of affection and may walk upon and over fellow monkeys as if they were part of the floor or an obstacle rather than a fellow being (MacLean, 1990; Joseph, 1996). Autistic children act as if other people are not present or are of no special interest (Fein et al., 1986) and move among other children as if among furniture (Kanner, 1943).

Hippocampus

As stated above, pyramidal neurons in some areas of the hippocampus of autistic individuals lack the complexity of dendritic branching, compared to normal controls. Although the hippocampus is mostly related to cognitive functions such as memory and attention, it is richly interconnected with other limbic structures that are of utmost importance in the expression of emotions. The hippocampus was proposed as part of Papez' original circuit involved in the mechanism of emotion (Papez, 1937), although it is now believed that its role in emotional expression is minimal (Joseph, 1996). Nevertheless, the hippocampus is capable of exerting influence and control upon other limbic structures. The hippocampus maintains an intimate relationship with the septal nuclei, which, in turn, exerts a major role in the control of emotions and the formation of long-term, lasting relationships. It is greatly influenced by its interactions with the amygdala in generating emotional imagery, encoding attention, learning, and memory (Joseph, 1996). In concert with the medial hypothalamus and septal nuclei, the hippocampus prevents extremes in arousal and maintains quiet alertness. The ability to modulate is frequently impaired

in autistic individuals, and malfunctioning hippocampal neurons may play at least a partial role in this behavioral dysfunction. In addition, the hippocampus has strong interactions with the amygdala in storing emotional reactions to events and recalling personal emotional memories, which seems to be impaired in autistic individuals. The hippocampus has a central role in contextual fear conditioning (LeDoux, 1996, 2000). The entorhinal cortex, which is abnormal in autistic individuals (Bauman and Kemper, 1994, 2005), is commonly considered as an integral component of the hippocampal formation (Nieuwenhuys et al., 2008) and forms part of a single functional complex, designated as the medial temporal lobe memory system (Squire and Zola-Morgan, 1991; Squire et al., 2004).

Cerebellum

Until very recently, the traditional view on the function of the cerebellum had been that it was solely involved in motoric functions (movement, motor control, coordination, balance, equilibrium, and motor learning). The cerebellum seemed to be the least likely area of the brain to be implicated in autism (DeLong, 2005). Interestingly, a body of literature of anecdotal and experimental evidence for non-motor functions of the cerebellum, dating back to the 19th century, had been largely ignored and had not been incorporated into mainstream neuroscience or clinical neurological thinking. The possibility of a causal relationship between cerebellar dysfunction and cognitive/psychiatric pathology was not considered or summarily dismissed. However, reports of aberrant behavior and cognitive deficits/mental retardation in subjects with cerebellar psychopathology had appeared in the literature starting as early as 1831 (Schmahmann, 1997). In 1950 Ray S. Snider saw the cerebellum as "the great modulator of neurologic function" and predicted its role in psychiatry (Snider, 1950). His later work on connections linking the cerebellum with the locus ceruleus and limbic structures – hippocampus, septum, and amygdala-further supported his argument for a need to reformulate the role of the cerebellum (Schmahmann, 1997).

In the 1970s, Heath carried on a series of electrophsysiological experiments that showed the functional connection of the cerebellum with the limbic system. By implanting electrodes in psychotic patients and experimental animals, he was able to demonstrate that when septal regions were active during pleasure feelings, activity in the hippocampus and amygdala was decreased. On the other hand, when pain and fear were being experienced, the activity in the hippocampus and amygdala was increased and septal activity was reduced (Heath, 1975). Heath demonstrated that the fastigial nucleus of the cerebellum was connected with the septum as well as with the hippocampus and amygdala. Recordings from electrodes implanted in the fastigial nucleus of an emotionally disturbed patient (Heath et al., 1974) showed that increased neuronal discharges correlated with the patient's experience of fear and anger. The amygdala, hippocampus, and septal nuclei – which show neuropathological abnormalities in the brain of autistic individuals – are under the modulation

of the fastigial nucleus which is also abnormal in autism. One may hypothesize that this modulatory control of limbic structures exerted by the cerebellar deep nucleus is deranged in autistics, resulting in an abnormal neural circuit of emotional expression and control.

In another series of experiments, Heath subdurally implanted electrodes in the vermis of patients with various psychiatric disorders, who had severe emotional dyscontrol. Amelioration of aggression was obtained in a great majority of patients by electrical stimulation. Heath attributed these effects to the connection of the vermis to the limbic system (Heath, 1977). Autistic individuals have been found in some studies to have abnormalities in the cerebellar vermis (although this finding is controversial), pointing to the possibility that the modulation of anger exerted by the cerebellum in normal individuals may be impaired in some individuals with autism, and may explain, at least in part, the lack of impulse control exhibited by some autistic persons.

The cerebellum also appears to be involved with the processing of emotional facial expression, which, as previously stated, appear to be deficient in autistic patients. In a functional MRI study, Critchley and collaborators studied high-functioning autistic individuals processing emotional facial expressions. Compared to controls, the fusiform gyrus (the cortical face area) as well as the left amygdala and the left cerebellum were not activated during processing emotional facial expressions. Cerebellar activity appears to be part of the circuit involved in processing facial emotion in normal subjects, but fails to be activated in autistic individuals (Critchley et al., 2000).

Schmahmann (Schmahmann, 2004; Schmahmann and Sherman, 1997, 1998) has coined the term "cerebellar cognitive affective syndrome" to denote a conglomerate of abnormalities seen in patients with cerebellar lesions of multiple etiologies. Cognitive and emotional psychopathology in these patients includes an impairment of working memory, planning, set shifting, verbal fluency, abstract reasoning, perseveration, logical sequencing, and bland or frankly inappropriate affect. Decrease in spontaneous conversation, telegraphic output, agrammatic speech, abnormal syntactic structure, and unusual prosody have also been noted in these patients. The cognitive and affective symptoms described in the cerebellar cognitive affective syndrome are also usually encountered in patients with disorders of the cerebellar hemispheres – association areas, paralimbic regions, and subcortical areas connecting these. The pontocerebellar system receives a considerable amount of input from limbic-related cortices, a plausible anatomic substrate for the role of the cerebellum in the modulation of affect (Schmahmann, 2004; Schmahmann and Sherman, 1997, 1998). According to Schmahmann (1994), the absence of motor abnormalities in autism does not imply that cerebellar neuropathology does not have clinical consequences, but rather that cerebellar abnormalities may result in disturbances in a neural network involved in the modulation and organization of emotion, language social interactions, and appropriate psychological behavior. The cerebellar nuclear abnormalities may play a role in the affective disturbances in autism, whereas the lateral and inferior cerebellar neuropathology may be related to abnormal language development and inappropriate social and psychological behaviors.

Developmental Hypothesis

The ontogeny of emotional and attachment behavior evident during the first year of life can be conceptualized heuristically in a coherent hypothetical biopsychosocial developmental model. According to this hypothesis, autistic infants often fail to achieve these developmental milestones from very early on, reflecting impairments in the development of limbic structures and their connections.

Kagan and Baird (2004) postulate the existence of five maturational transition periods, two of which occur during the first year of development. The first maturational transition period takes place at 2–3 months of age in the human infant. This is manifested by the disappearance of newborn reflexes through the cortical inhibition of brainstem neurons. New synaptic contacts appear together with growth of inhibitory neurons in the spinal cord. This coincides with the reduction in crying and increases in social smiling, which may be attributable to the cortical inhibition of brainstem nuclei (e.g., the periacqueductal grey matter) that mediate crying. At the same time the infant develops the ability to recognize an event following a delay and the possibility of establishing visual expectation, which coincides with the greatest increase in hippocampal growth rate of mossy cells in the dentate gyrus. According to Joseph (1996) the period around 6–7 months of age corresponds to the amygdaloid maturation period. The normal infant increasingly seeks social, physical, and emotional contact. This is the period of indiscriminate contact seeking, during which the infant smiles at strangers. The second transition occurs at 7–12 months in healthy infants (Kagan and Baird, 2004). This is accompanied by the capacity to retrieve schemata from past events that are no longer in the perceptual field and to hold them along with the current perception in a working memory circuit. This coincides with separation fear. Upon mother's departure, the infant retrieves schemata from the former presence of mother to the current perception of her absence. If unable to assimilate this disparity, the infant becomes fearful and cries. The normal infant also develops fear of strangers when an unfamiliar face does not conform to the schemata of the caretaker's face. Limbic structure maturation parallels this transition phase. Hippocampal volume approaches adult size between 10 and 12 months. The integrity of the hippocampal formation is necessary for holding a representation in a short-term memory store for less than 10 s. The amygdala is activated by the above events and projections from the amygdala to the cortex are myelinized between 7 and 10 months, coinciding with the emergence of fear of strangers and separation anxiety (Kagan and Baird, 2004). Septal and cingulate development is prominent around 7–8 months of age. The infant becomes more discriminating. Real and specific attachments are formed to caretakers. Attachments become progressively more intense and stable. Septal and cingulate development continues in normal infants during the 7–12 months period. Indiscriminate social contact seeking, which may be attributed to a relatively unopposed amygdala, is inhibited, whereas specific attachments, which may be attributed to the developing septal nuclei and anterior cingulate gyrus, are strengthened, reinforced, and maintained (Joseph, 1996).

Autistic infants fail to achieve this progressive level of human attachment, manifested by virtual lack of regard for the caretakers' presence, decreased or absent

social smiling, apparent lack of desire to be held, and lack of separation anxiety and fear of strangers. This reflects a derangement in the maturation of limbic structures, specifically the amygdala, septal nucleus, hippocampus, and anterior cingulate, which have been shown to be abnormal in autism (Bauman and Kemper, 1994, 2005; Schumann and Amaral, 2006).

MacLean (1985, 1990) has hypothesized that the history of the evolution of the limbic system is the history of the evolution of mammals. The deficits in the limbic structures of autistic individuals appear to ontogenetically mirror a phylogenetic deficiency in paleomammalian brain evolution. Behaviors at the basic core of autistic symptomatology – the expression of affection, social–emotional reciprocity, attachment behaviors, and the capacity to play – that are impaired in individuals with autism represent behaviors that evolved during the transition from reptiles to mammals. These abnormalities of limbic structures have profound human consequences in what is one of the most severe psychiatric disorders and undermine the essence of personality qualities we value as being human.

Conclusions

This chapter has selected limbic and cerebellar structures that have consistently been found in neuropathological studies to be abnormal in autism, and attempted to formulate a theoretical model or hypothesis about how these abnormalities correlate with psychopathology – especially emotional and attachment problems – in patients with autism. For the sake of clarity, these structures have been discussed separately, and their complexity simplified. Nevertheless, the fact that these structures do not operate independently, but interact in a very complex manner that is beyond our present comprehension, should not be overlooked. As the subtitle indicates, the ideas expressed in this chapter are only a working hypothesis formulated with the aim of stimulating thinking, discussion, questioning, agreement or disagreement, acceptance or rejection, and hopefully advancing and moving up to higher levels of neurobehavioral analysis.

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The Morphology of Minicolumns

Alan Peters

Minicolumns

The cerebral neocortex is a thin sheet of gray matter covering the cerebral hemispheres, and it is composed of six horizontally dispersed layers of neurons. Throughout the cerebral hemispheres these layers vary in thickness, sizes of their neurons and packing density, and these differences have been used to subdivide the cerebral cortex into regions and areas (e.g., Brodmann, 1909). However, despite the horizontal layering, functionally it is the vertical connections between neurons that are of prime importance, because as first expounded by Lorente de Nó (1938), the vertical connections between neurons in the cortex are stronger than the horizontal ones. Subsequently Mountcastle (1978) proposed that cortical neurons are organized into narrow, vertically interconnected, units that extend through layers 2–6, and he suggested that these units should be called "minicolumns," although they are also often referred to as "microcolumns." As recently pointed out by Jones (2000), the minicolumn hypothesis "requires that neurons in the middle layers of the cortex, in which thalamic afferents terminate, should be joined by narrow vertical connections to cells lying superficial and deep to them, so that all cells in the column are excited by incoming stimuli with only small latency differences."

The minicolumns can be demonstrated physiologically by experiments in which a microelectrode is inserted into the cortex in an essentially horizontal direction. When this is done, it is found that there are changes in the receptive properties of the cortical neurons every 50 μm or so, as the electrode passes from one minicolumn into the next one, and that groups of these microcolumns are activated by peripheral stimuli to generate larger units, the macrocolumns, or functional columns. The question that has produced debate is what is the anatomical equivalent

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of the physiologically defined microcolumns? Basically there are two organizational arrays of neuronal components that have been suggested as possible candidates and, as will be shown, morphologically these arrays are related to each other. One array is three-dimensional, while the other is two-dimensional, and the sizes of both of these arrays are commensurate with those of the physiologically defined minicolumns. The three-dimensional vertical arrays have at their centers, or axes, apical dendrites of pyramidal cells that come together to form "clusters," and the pyramidal cells associated with these arrays form "pyramidal cell modules.". The axons of the neurons in these modules also aggregate to form vertically oriented bundles that pass through the deeper layers of the cortex to enter the white matter. Studies of these pyramidal cell modules have generally focused on their organization, development, and physiological functioning in the cortex.

The two-dimensional arrays of neurons that are visible in Nissl stained sections are vertical stacks or strings of neuronal cell bodies that are most obvious in the upper layers of the cortex. These strings of neurons are better defined in some cortices, such as the primate temporal lobe, than in others. Studies of these entities have largely relied upon digitized images and have focused on ascertaining if there are changes in the spacing of neurons in these arrays in various disease states and in cortices of patients with behavioral problems. In this review we will consider how these two anatomically defined entities are related to each other. For recent reviews that have considered the composition, function, and significance of minicolumns those of Jones (2000), Buxhoeveden and Casanova(2002a and 2002b), and Rockland and Ichinohe (2004), and the book edited by Casanova (2005), should be consulted.

Physiologically Defined Units

A more complete account of the physiologically defined minicolumns can be found in the review by Mountcastle (1997) and in his book on cerebral cortex (Mountcastle, 1998). The sizes of the physiologically defined minicolumns are derived from experiments such as those of Favorov and Whitsel (1988), and Favorov and Diamond (1990), who showed that when electrode penetrations of the somatic sensory cortex of the cat are made slightly off vertical, and the skin stimulated, there is a abrupt shift in receptive field properties of the cortical neurons about midway through the depth of the cortex. Such shifts occur with lateral movements of the recording electrode every 40–50 μm, which indicates that the electrode is moving from one minicolumn to the next one.

Another example of an experiment showing the sizes of the physiologically defined minicolumns is the nerve regeneration study carried out by Kaas et al. (1981). These experimenters made an initial electrode penetration of the somatic sensory hand area in a monkey's neocortex in a direction more or less parallel to the surface of the cortex and showed that over a considerable distance the same modality type is observed. They then sectioned the median nerve and allowed time for the nerve to regenerate and re-innervate of the skin. The recording experiment was then

repeated, and it was found that instead of the smooth progression of overlapping receptive fields, sharp shifts in the receptive fields occurred every 40–60 μm. It was proposed that the reason for this change is that during re-innervation of the skin, the nerve fibers of the medial nerve become misguided and are not able to find their way back to their original postsynaptic loci. Consequently a new receptive field distribution is imposed on the entire system of minicolumns. Other examples showing the sizes of the physiologically defined minicolumns can be found in the reviews by Mountcastle (1997; 1998) and Buxhoeveden and Casanova (2002a).

Pyramidal Cell Modules: Anatomically Defined Units Based on Apical Dendrite Clustering

Although the early work of Lorente de Nó (1938) had pointed out that the connectivity between neurons in the cerebral cortex is essentially vertical, it was not until 1972 that vertically organized morphological units of cortical neurons were shown to exist in cerebral cortex. Using light and electron microscopy of plastic-embedded material, Peters and Walsh (1972) demonstrated that in the somatosensory cortex of the rat, groups of apical dendrites of layer 5 pyramidal cells become clustered together, and as they ascend through the cortex and enter layer 3, the apical dendrites of pyramidal cells in that layer are added to the peripheries of the layer 5 clusters. Simultaneously Fleischhauer et al. (1972), using a number of light microscopic stains in combination with electron microscopy showed a similar clustering of apical dendrites in the sensory–motor cortex of the rabbit and cat. Peters and Walsh (1972) found that the center-to-center spacing of the apical dendritic clusters was about 50 μm and Fleischhauer et al. (1972) gave the sizes of the clusters as about 40–50 μm. Both groups of investigators proposed that the vertically arranged groups of neurons with clustered apical dendrites are the anatomical equivalents of the physiological defined minicolumns, the clustered apical dendrites being the centers, or axes, of the minicolumns.

Since the initial descriptions of the existence of clusters of apical dendrites, other anatomical studies have been carried out, and it is now evident that such clustering of apical dendrites occurs throughout the neocortex (see Table 1). Thus clusters of apical dendrites have now been directly visualized and described in such locations as primary visual cortex of the rat (Winkelmann et al., 1975; Peters and Kara, 1987); mouse posteromedial barrel field (White and Peters, 1993) and primary motor cortex (Lev and White, 1997); the somatosensory cortex (Massig and Fleischhauer, 1973) and visual and motor cortex of the rabbit (Schmolke and Fleischhauer, 1984; Schmolke and Viebahn, 1986; Schmolke, 1987); cortical areas 2, 3, 4, and 41 of the rat (Feldman and Peters, 1974); the primary auditory, primary visual, and postcruciate cortex of the cat (Feldman and Peters, 1974; Peters and Yilmaz, 1993); primary visual cortex of the monkey (Peters and Sethares, 1991; 1996); and the cerebral cortex of the lesser Madagascan hedgehog and red-eared pond turtle (Schmolke and Künzle, 1997).

Table 1 Center-to-center spacing of dendritic clusters in neocortex

Species	Area studied	Center-to-center spacing (μm)	Authors
Rat	Somatic sensory cortex	50	Peters and Walsh (1972)
Rat	Somatic sensory cortex	49	Skoglund et al. (2004)
Rat	Area 17	30–40	Feldman and Peters (1974)
Rat	Area 17	76	Winkelmann et al. (1975)
Rat	Area 17	55–60	Peters and Kara (1987)
Rat	Visual cortex	53	Lohmann and Koppen (1995)
Rat	Visual cortex	27	Vercelli et al. (2004)
Rat	Area 3	50	Feldman and Peters (1974)
Rat	Area 41	50–70	Feldman and Peters (1974)
Rat	Prelimbic cortex (area 32)	44	Gabbott and Bacon (1996)
Mouse	Parietal region	50–100	Escobar et al. (1986)
Mouse	Sm1 barrel subfield	22–25	White and Peters (1993)
Mouse	MsI	31	Lev and White (1997)
Hedgehog tenrec	Areas A2, A3, and A4	32	Schmolke and Kunzle (1997)
Rabbit	Sensory motor	40–50	Fleischhauer et al. (1972)
Cat	Area 41	50–70	Feldman and Peters (1974)
Cat	Area 17	56	Peters and Yilmaz (1993)
Monkey	Area 17	31	Peters and Sethares (1991)
Monkey	Area 17	23	Peters and Sethares (1996)
Monkey	Area 18	21	Peters et al. (1997)
Human	Medial prefrontal cortex	52–59	Gabbott (2003)

The Basic Structure of the Pyramidal Cell Modules

Perhaps the best-understood and clearest example of dendritic clustering is found on the rodent primary visual neocortex, in which neurons are stratified into six obvious layers. In this cortex, pyramidal cells occur in all layers except layers 1 and 6b, and they account for 85–90% of all neurons (Peters and Kara, 1985; Gabbott and Stewart, 1987). The majority of apical dendrites of the pyramidal cells in layer 5 are aggregated into clusters as they ascend through the cortex to form their apical tufts in layer 1. As these clusters of layer 5 apical dendrites ascend, the apical dendrites of the pyramidal cells in layer 2/3 are added to them. This information was first obtained by tracing apical dendrites using serial semi-thick plastic sections, but subsequently a less painstaking way to demonstrate this arrangement became available when antibodies were produced against microtubule-associated protein 2 (MAP2), which occurs in the cell bodies and dendrites of neurons (de Camilli et al., 1984). Use of this antibody clearly reveals the bundles of pyramidal cell apical dendrites when they are sectioned either along their length (Fig. 1) or sectioned transversely (Fig. 2). The mean center-to-center spacing of the apical dendritic clusters in rat visual cortex is 55–60 μm, and on average each cluster contains the apical dendrites of 12 large- and 38 medium-sized layer 5 pyramidal cells (Fig. 3; Peters and Kara, 1987; Peters, 1993). However, the apical dendrites of some layer 5 pyramidal cells appear not to contribute to the clusters; instead they ascend either singly or in pairs

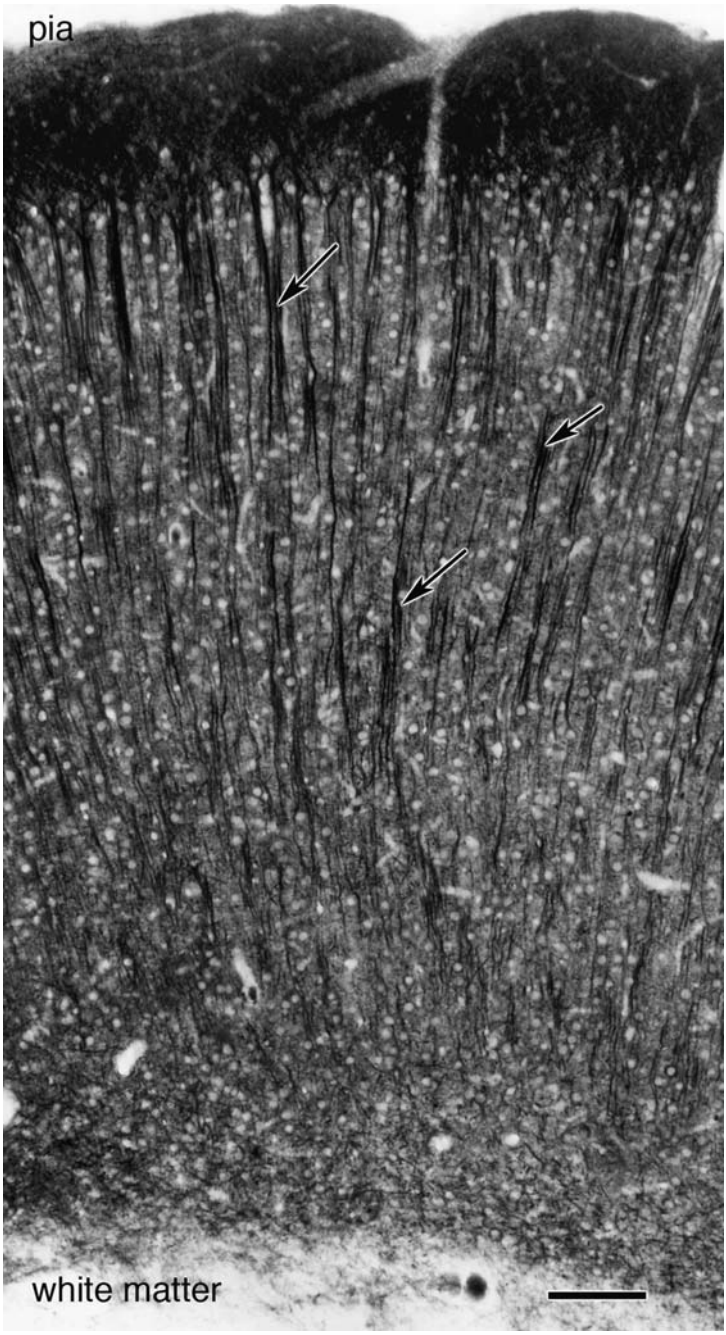

Fig. 1 Rat visual cortex sectioned in the vertical plane and labeled with an antibody to MAP2 to show the apical dendritic clusters (*arrows*). Note the densely stained layer 1 beneath the pia, and the pale staining of the white matter. Scale bar = 100 μm

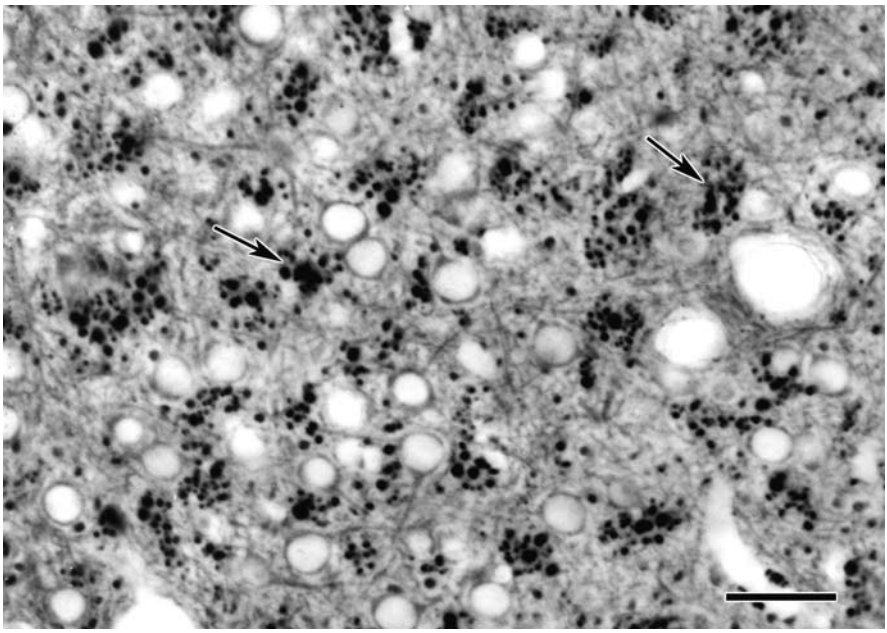

Fig. 2 Transverse section of rat visual cortex at the level of layer 4. The section is labeled with MAP2 antibody to show the darkly stained apical dendrites within the clusters (*arrows*). Scale bar = 50 μm

as they enter layer 4, and whether they join the clusters as they ascend through upper layers has not been ascertained.

As first shown by Escobar et al. (1986) in the neocortex of the mouse, the apical dendrites of the pyramidal cells in layer 6a do not join the clusters formed by the apical dendrites of the pyramidal cells in layers 5–2. Instead they form their own independent groupings, which we have referred to as "bundles." Since the pyramidal cells of layer 6a are small, their apical dendrites are thinner than those of the pyramidal cells in upper layers and they form numerous bundles that ascend as far as layer 4, where they terminate and form their apical tufts (see Figs. 3 and 4).

A comment needs to be made about the difference in measurements of the center-to-center spacing of these dendritic bundles in rat visual cortex as determined by various investigators. As seen in Table 1, most of the measurements of center-to-center spacing range between 40 and 60 μm. At the high end is the value of 76 μm obtained by Winkelmann et al. (1975), and at the lower end the value of 27 μm obtained by Vercelli et al. (2004). The reason for these large differences is yet not fully apparent, but Vercelli et al. (2004) suggest that it might be due to the fact that most investigators have only determined the frequency of occurrence of counted dendritic clusters at the level of layer 4, and have concentrated only on clusters that contain large diameter dendrites. Curtetti et al. (2002) point out that there are

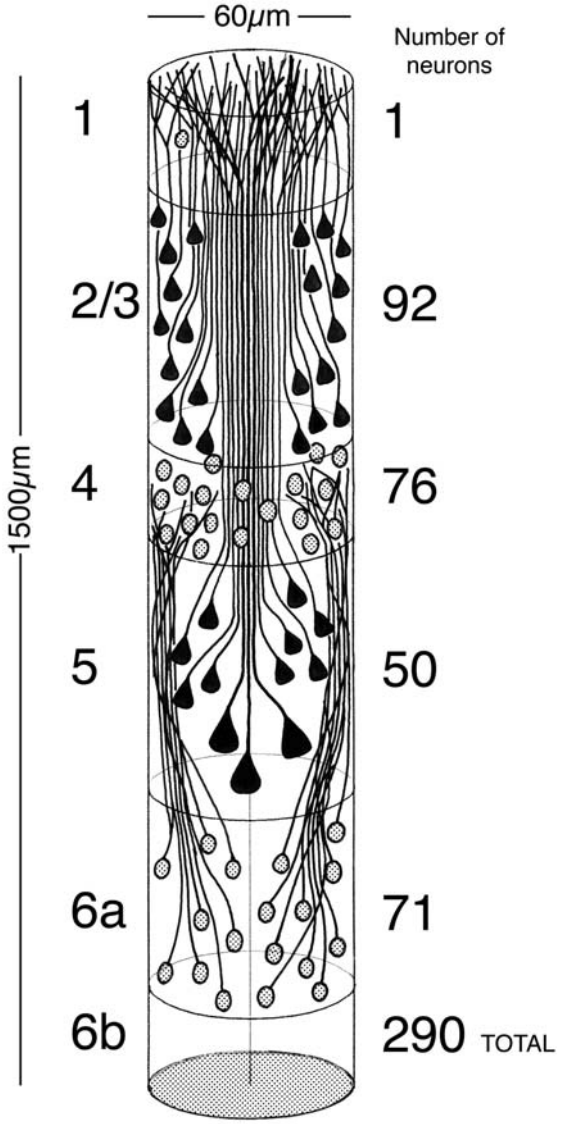

Fig. 3 Diagrammatic representation of the pyramidal cell module in rat primary visual cortex, area 17. The cortical layers are indicated on the *left* and the number of neurons in each layer contributing to the module is given on the *right*. After Peters (1993)

several types of apical dendritic clusters, some consisting of apical dendrites that arise exclusively from layer 5, others that arise from pyramidal cells in layers 5, 3, and 2, and yet others that are formed from neurons in the supragranular layers. Vercelli et al. (2004) state that they included all of these types of dendritic clusters in their analyses, so that their value for the number of clusters per unit area of tangential sections is greater than that obtained by previous authors. But as will be noticed in Table 1, the value obtained by Vercelli et al. (2004) is very similar

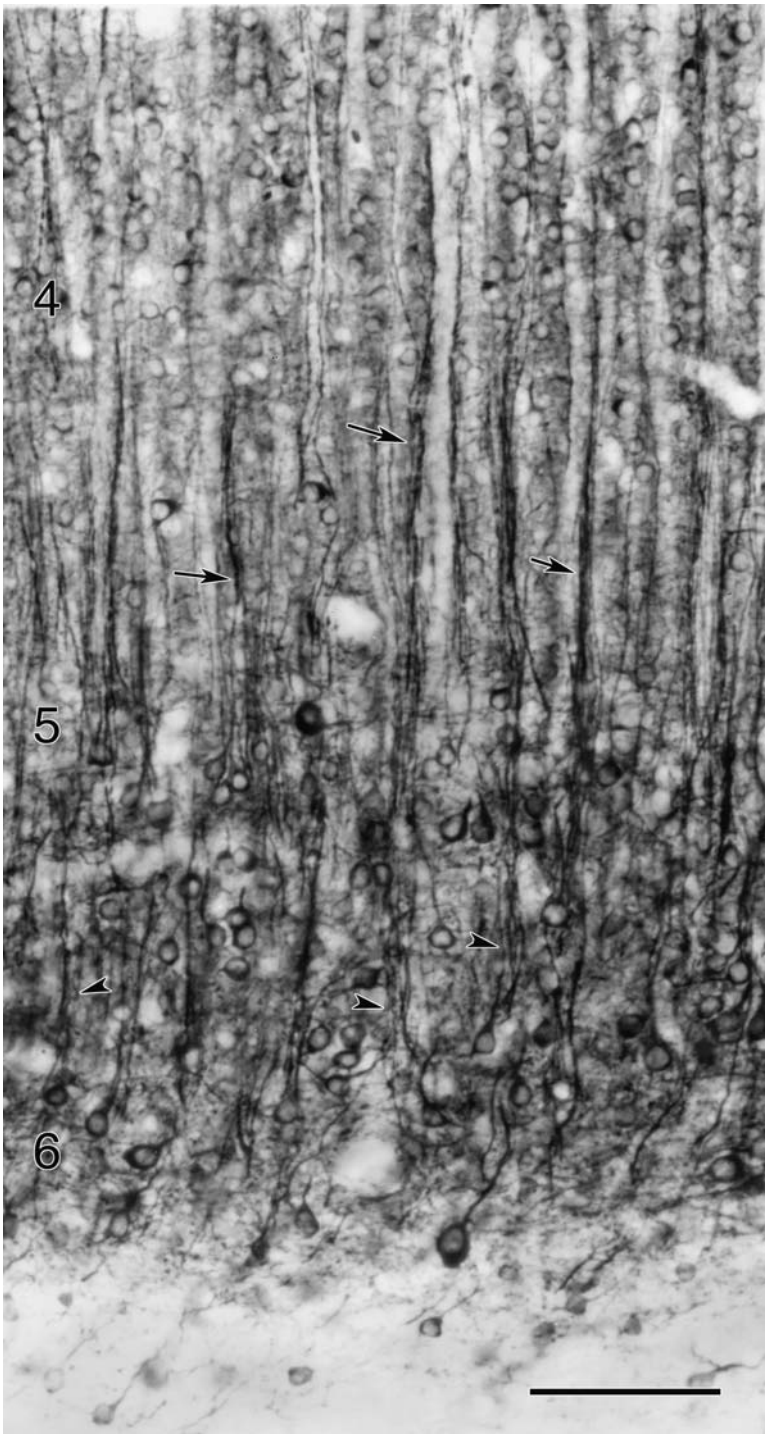

Fig. 4 (continued)

to that obtained by White and Peters (1993) in mouse barrel field and by Lev and White (1997) in mouse motor cortex. However, it should be noted that White and Peters (1993) found some variation in the spacing of apical dendritic clusters, in that the clusters in the walls of barrels have an average spacing of 22 μm, while in the barrel hollows their average spacing is 25 μm. The reason is that, as shown by Detzer (1976), apical dendrites at the periphery of the hollows in mouse barrel field often bend, or become deflected, as they approach layer 4, so that they enter the walls of the barrels, thereby increasing the concentration of clusters in the barrel walls.

Apical Dendritic Clusters in the Neocortices of Other Animals

In other cortices there is a similar aggregation of apical dendrites of layer 5 pyramidal cells into clusters and the aggregation of apical dendrites of layer 6a pyramidal cells into bundles. This is true for the organization of pyramidal cell apical dendrites in sensory–motor cortex of the rabbit (Fleischhauer et al., 1972), areas 41 and 17 of the cat (Feldman and Peters, 1974; Peters and Yilmaz, 1993), area 17 of the monkey (see Figs. 4 and 5; Peters and Sethares, 1991; 1996), and medial prefrontal cortex of the human brain (Gabbott, 2003). The one exception appears to be area 18 of the monkey, in which apical dendrites of layer 5 pyramidal cells aggregate with those from layer 6a pyramidal cells to form swathes of apical dendrites that ascend into layer 4, where they are joined by the apical dendrites of layer 4 pyramidal cells (Fig. 5). In area 18 the majority of the apical dendrites from layers 6a, 5, and 4, form their apical tufts in layer 4 and lower layer 3, with only a few of them ascending as far as layer 1. Consequently, it is the apical tufts of layer 2 and 3 pyramidal cells that dominate layer 1. Thus, in area 18 of monkeys, there are essentially two separate tiers of apical dendrites: a lower tier containing apical dendrites from pyramidal cells in layers 6a–4 and an upper tier containing apical dendrites of layer 2/3 pyramidal cells (Fig. 5).

Another interesting variation in the clustering of apical dendrites occurs in the cat sensorimotor cortex. There it has been shown by Fleischhauer (1974) that in the posterior sigmoid gyrus the apical dendrites of the layer 5 pyramidal cells frequently bifurcate soon after they emerge from the large cell bodies. The secondary dendritic branches then run obliquely for a short distance and at the level of layer 3 they join secondary branches from other apical dendrites to form dendritic clusters

Fig. 4 (continued) Monkey primary visual cortex. Vertical sections labeled with an antibody to MAP 2. The illustration shows the apical dendritic clusters (*arrows*) arising from layer 5 pyramids and the bundles of apical dendrites (*arrowheads*) arising from layer 6a pyramidal cells. The locations of layers 4, 5, and 6 are indicated. From Peters and Sethares (1991). Scale bar = 100 μm

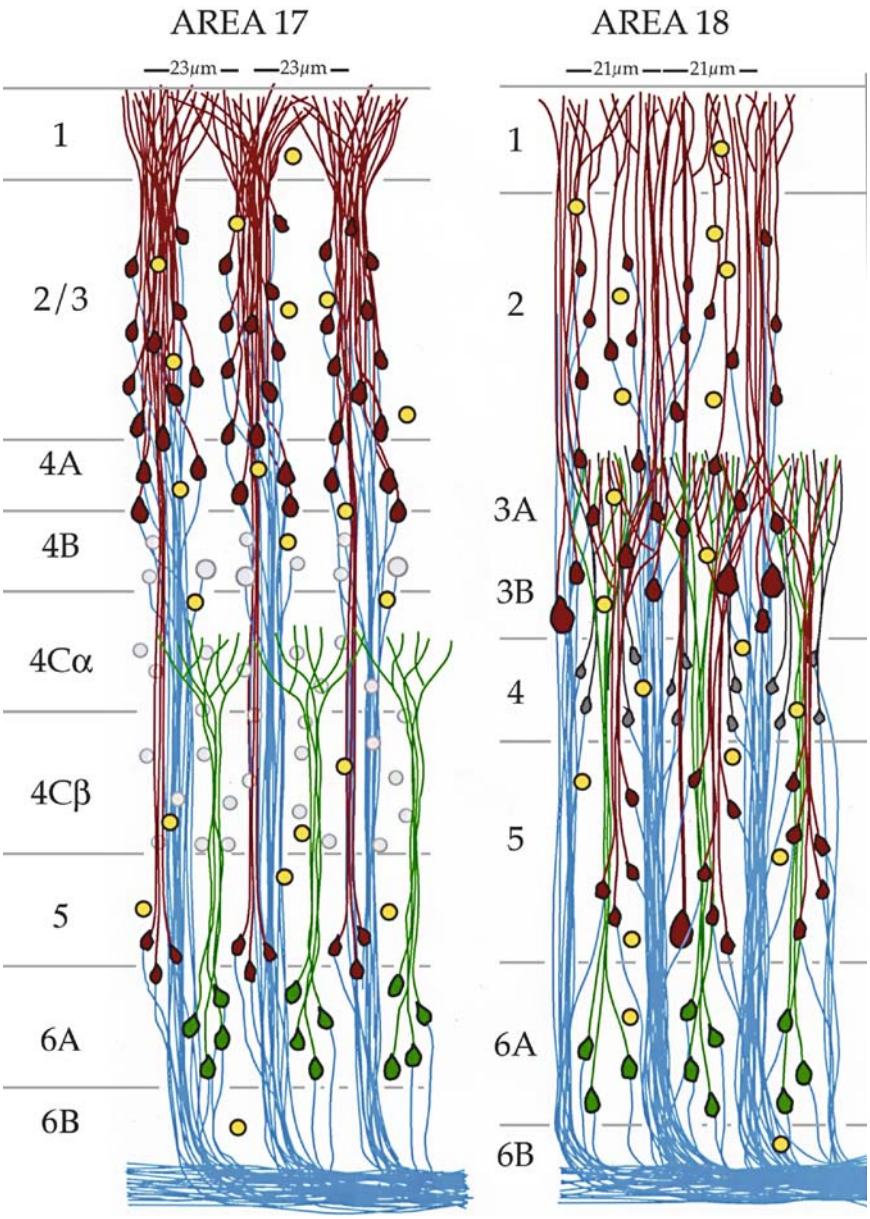

Fig. 5 Diagram to show the differences in the arrangement of pyramidal neurons in areas 17 and 18 of monkey visual cortex. Not all of the neurons in a module are shown in the diagram. The pyramidal cells in layers 5, 4A, 3, and 2 are shown in *red*, and the pyramidal cells in layer 6A are in *green*. Neurons in layer 4 are *grey*, while inhibitory neurons are *orange*. The bundles of myelinated nerve fibers that extend from the modules to enter the white matter are shown in *blue*.

that extend up to layer l. A similar bifurcation of layer 5 apical dendrites, with secondary branches entering neighboring clusters, has also been noted by Massig and Fleischhauer (1973) in the somatosensory cortex of the rabbit.

The Spatial Arrangement and Connections of Apical Dendritic Clusters

Peters and Kara (1987) proposed that dendritic clusters are basically arranged in a hexagonal pattern. The pattern is not perfect, but in tangential sections through rat primary visual cortex taken at the level of layer 4, most dendritic clusters appear to be at the center of six other clusters. This same distribution pattern is also found in monkey primary visual cortex (Peters and Sethares, 1996); in cat primary visual cortex (Peters and Yilmaz, 1993); prelimbic cortex of the rat (Gabbott and Bacon, 1996); and in Brodmann areas 25, 32, and 24 of the human medial prefrontal cortex (Gabbott, 2003). However, after carrying out a mathematical analysis of the distribution of dendritic clusters in primary somatosensory cortex of the rat, Skoglund et al. (2004) concluded that there is not a hexagonal pattern of cluster distribution in this cortex, since the distances between individual clusters range between 24 and 121 μm.

Obviously, the question of the geometric distribution of dendritic clusters needs to be examined further, and any modular theory of cortical function based on the concept that the apical dendritic clusters are the axes of functional cortical modules, or minicolumns, has to take into account that there is biological variation in the composition of the modules, as shown by studies like those of Lev and White (1997) and Vercelli et al. (2004).

Lev and White (1997) labeled callosally projecting neurons in MsI cortex of the mouse by retrograde transport of horseradish peroxidase deposited onto severed callosal fibers in the contralateral hemisphere. They found that in some dendritic clusters in the contralateral hemisphere all of the apical dendrites were labeled, whereas in adjacent clusters none of the dendrites were labeled. This suggests that some pyramidal cell modules are composed exclusively of callosally projecting neurons, while other clusters have different functions. Vercelli et al. (2004) examined the output neurons in rat visual cortex using lipophilic tracers to label different pyramidal cell populations and determined that neurons contributing to dendritic clusters can have different specific targets. Pyramidal cells projecting to ipsi- and contralateral cortex cluster together and the same clusters contain neurons that project to

Fig. 5 (Continued) Note that in area 17 the apical dendrites of layer 5 pyramids pass into layer 2/3 where they are joined by the apical dendrites pyramidal cells in that layer, but the bundles of apical dendrites of the layer 6A pyramidal only extend as far as layer 4C. In area 18, the apical dendrites of the layer 5 and layer 6A pyramidal cells both extend only up to layer 3A. Nearly all of the apical dendrites in the clusters in the upper layers of the cortex arise for the layer 2 and 3 pyramidal cells. From Peters et al. (1997)

the striatum. But the clusters that contain callosally projecting cells do not contain neurons that project to the superior colliculus, the dorsal division of the lateral geniculate body, or project through the cerebral peduncle. Consequently not all pyramidal cell modules are identical and there is not a random mixing of projection neurons, but some degree of specificity.

Myelinated Axon Bundles

Although they are not very evident in rodent cortex, vertical bundles of myelinated axons are prominent in cortices such as those of the cat and primates. These vertical bundles of myelinated axons generally become evident at the level of layer 3 and extend through the depth of the cortex, ultimately blending with the myelinated nerve fibers in the underlying white matter. In the primary visual cortex (Peters and Sethares, 1996) and in area 18 (Peters et al., 1997) of the rhesus monkey cortex, it has been shown that these vertical bundles of myelinated axons arise from the groups of pyramidal cells whose apical dendrites aggregate together to form the clusters. It is assumed that each vertical bundle of myelinated axons represents the output or efferent fibers from the neurons associated with individual clusters of apical dendrites. Consequently, it is not surprising to find that the center-to-center spacing of the vertical bundles of myelinated axons is the same as that of the apical dendritic clusters within the same cortical area (Figs. 5 and 6).

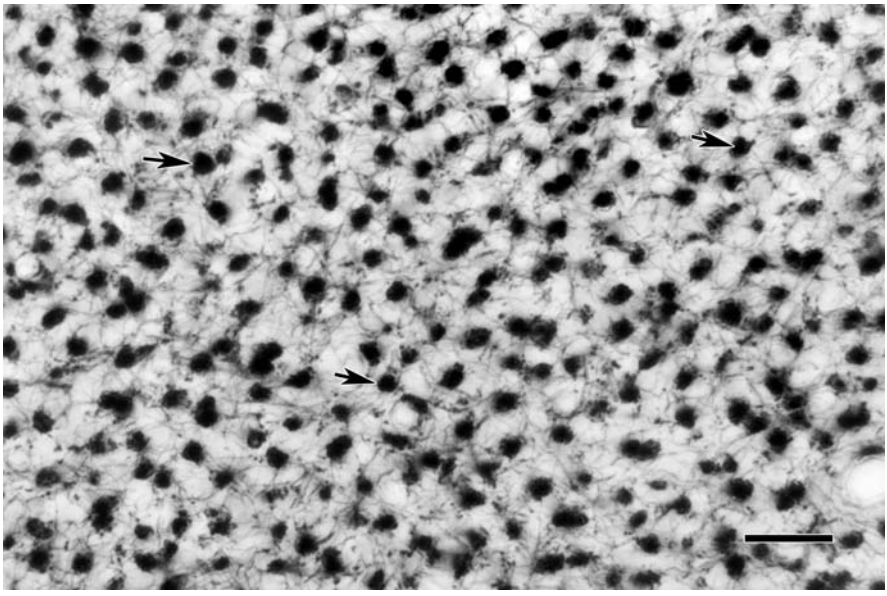

Fig. 6 Monkey primary visual cortex transverse section taken at the level of layer 5 and stained to show the regularly spaced bundles of myelinated axons (*arrows*). Scale bar = 25 μm

Lohmann and Köppen (1995) have also shown that the dendritic clusters and the vertical axonal bundles in rat visual cortex originate from the same neurons, and that the axonal bundles and the dendritic clusters have similar center-to-center spacing. Recently Casanova et al. (2008) have concluded that in the human cortex the pyramidal cell arrays and the vertical bundles of myelinated axons have similar spacing.

Unmyelinated Axon Bundles

In addition to vertical bundles of myelinated axons, the cerebral cortex of monkeys (e.g., DeFelipe et al., 1990) and of humans (e.g., del Rio and DeFelipe, 1995) also contains vertically oriented bundles of unmyelinated axons that are referred to as horsetails. These horsetails are the axonal plexuses of the inhibitory double bouquet cells and can be demonstrated in monkey neocortex by immunolabeling with antibodies to calbindin and tachykinin. As shown by DeFelipe et al. (1990), in the monkey these axonal bundles are widespread and form a regular columnar system descending from layer 2 to layers 3–5. The bundles are most evident in tangential sections taken at the level of layer 3, where they can be seen to have a center-to-center spacing of 15–30 μm. In a later study of the calbindin labeled double bouquet cells in monkey striate cortex, Peters and Sethares (1997) showed that there is one double bouquet cell, and therefore one vertically oriented double bouquet cell axonal plexus, or horsetail, per pyramidal cell module (Fig. 7). Within layer 2/3 the double bouquet axons run alongside the apical dendritic clusters, while in layer 4C they are closely associated with the vertical myelinated axonal bundles. DeFelipe et al. (1989; 1990) proposed that the axon terminals of the double bouquet cell synapse with the shafts and spines of basal dendrites and oblique shafts of apical dendrites of pyramidal cells, but the exact role of these vertical bundles of inhibitory axons is not known. It is likely that they constitute a vertical inhibitory system that acts upon pyramidal cells within the minicolumns.

Yanez et al. (2005) have carried out a survey of the distribution of double bouquet cells in the cortices of various mammalian species. There are no double bouquet cells in the neocortices of rodents and rabbits, and compared to primates there are relatively few double bouquet cells in the cortices of carnivores such as cats, dogs, lions, and cheetahs. Consequently there is great variation in the occurrence of double bouquet cells with horsetail axons, and Yanez et al. (2005) conclude that although double bouquet cells are an important neuronal element in the organization of minicolumns in primate neocortex, this is less true in other mammalian species.

A Conclusion

It is evident that the modules of pyramidal cells whose apical dendrites form clusters and the vertical bundles of myelinated axons are facets of the same basic, modular organization of neurons into vertical units that we can refer to as minicolumns. And

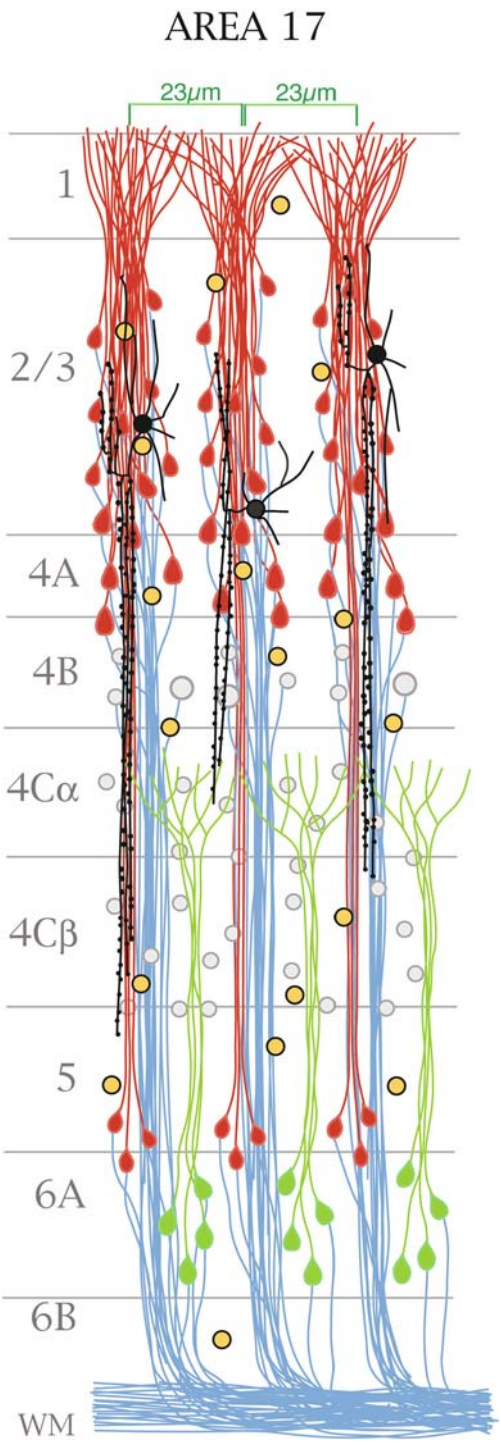

Fig. 7 Diagram of the microcolumn in monkey visual cortex to show that there is one double bouquet cell horsetail (*black*) per pyramidal cell module. The other colors correspond to those in Fig. 5, which explains the composition of the pyramidal cell modules in monkey area 17. From Peters and Sethares (1997)

furthermore, in primates the horsetail bundles of axons from double bouquet cells are strongly associated with the pyramidal cell modules.

As suggested by Peters and Sethares (1996) it is not likely that individual minicolumns have definite boundaries. For example, although the apical dendrites of pyramidal cells may be clustered, the basal dendrites of these same neurons extend for some distance laterally and intertwine with the basal dendrites of neurons in other clusters. In addition the axons of the thalamic input to the cortex have terminal plexuses that spread over several hundred microns, and the axonal plexuses from different thalamic neurons overlap each other. Nevertheless if the minicolumns are looked upon as being arranged in a two-dimensional sheet, even though the thalamic inputs to this sheet overlap, it is likely that an individual minicolumn receives an input that is slightly different from that received by its neighbors. Thus, in visual cortex, for example, the neurons in an individual minicolumn could respond to a slightly different part of the receptive field than the neurons in its neighboring minicolumns, and perhaps respond better to a different orientation of an image, and a different color or eye preference than its neighbors. Given these possibilities it may be expected that the output of the neurons in each minicolumn carried through its vertical bundle of myelinated nerve fibers, is unique to that minicolumn.

Minicolumns Based on Vertical Arrays of Neuronal Cell Bodies Seen in Nissl Stained Sections

In some areas of the cerebral cortex, and especially in the primate temporal lobe, vertical arrays, or strings, of neuronal cell bodies are evident in Nissl stained vertically oriented sections. The Nissl stained vertical arrays are one or two cell bodies wide and they are especially obvious in layers 2–4 (Fig. 8). Intervening between these vertical arrays of neuronal cell bodies are pale zones that presumably contain dendrites and axons, which have little Nissl substance. However, it should be borne in mind that the Nissl image is two-dimensional and how the cell bodies are arranged in three dimensions is not obvious from such sections. von Bonin and Mehler (1971) examined sections of human cortex cut obliquely, but basically in the tangential plane, and although their description is sketchy, it implies that the neurons are arranged in rather indistinct vertically oriented groups, strings or rows, that are as much as 80 μm apart. How this image fits with what is known about the arrangement of apical dendrites into clusters and myelinated axons into bundles is not fully evident, but it is likely that the pale spaces between the strings of neuronal cell bodies correspond to the clusters and bundles of apical dendrites. An issue that has not been resolved is how the cell bodies of cortical neurons are arranged. From studies of clusters it is generally assumed that the neuronal cell bodies surround the dendritic clusters, whereas Nissl preparations would suggest that the neurons are in discrete vertical strings. Which arrangement is correct, needs to be resolved by making three-dimensional reconstructions of the disposition of neuronal cell bodies through the depth of the cortex, and this has not yet been done.

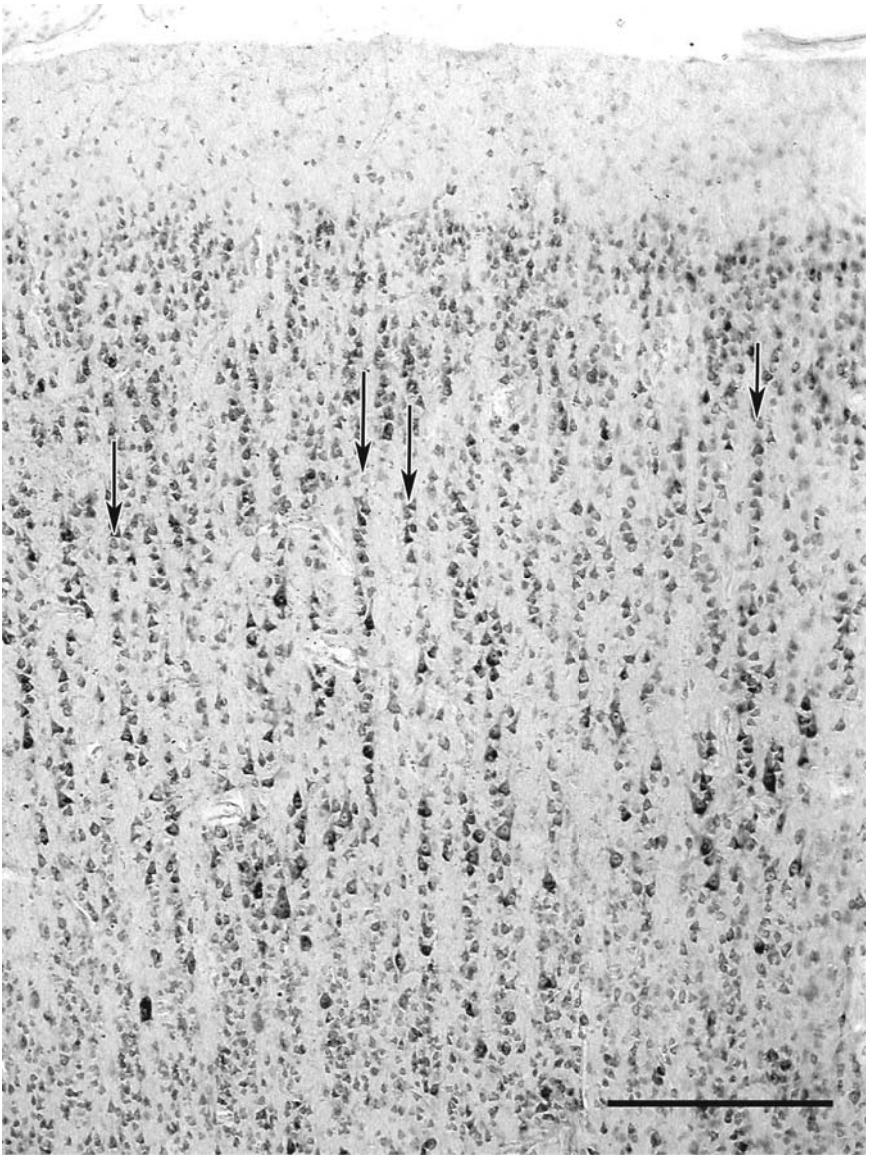

Fig. 8 Nissl stained section of primary auditory cortex, area 41, from a human cerebral hemisphere. In this cortical area there are obvious vertical strings of neurons (*arrows*) separated by pale spaces that are largely occupied by the apical dendrites of pyramidal cells. Scale bar = 100 μm

The only study in which both the disposition of dendritic clusters and of vertical arrays of neurons in the same cortical areas have been compared in the same cortical area is that of Gabbott (2003). He examined human prefrontal cortex using both MAP2 labeling to show dendritic clusters in sections cut parallel to the cortical

surface and in Nissl stained sections cut in the vertical plane to show the vertical strings of neuronal cell bodies. Gabbott (2003) found the center-to-center spacing of the dendritic clusters to be 52–59 μm, and the center-to-center spacing of the vertically oriented strings of neurons to be 49–60 μm, suggesting that the two entities are different views of the same neuronal organization in the cortex. However, Gabbott (2003) makes no comment about how the neuronal cell bodies are disposed.

One reason why more studies like that of Gabbott (2003) have not been carried out is that most studies of the composition and dimensions of dendritic clusters and pyramidal cell modules have been carried out in mice, rats, cats, and rabbits, with a few studies in monkeys and only one in humans (see Table 1). In contrast all of the studies of the dimensions of minicolumns based on spacing of vertical strings of neurons in Nissl stained sections have been carried out in monkeys, ape, and human cortex (see Table 2).

Typically, even in temporal cortex the vertical arrays of neurons are not always visible throughout the entire depth of the cortex, but they are generally obvious in layers 2 and 3 (see Fig. 8). Consequently it is in these layers that most analyses and measures of these vertical arrays of cells have been carried out using digitized images. Since this is a young field of research, the methods of analysis and the nomenclature used by various sets of investigators vary, making it sometimes difficult to compare the numerical data generated in various studies and to even discern what is meant by the term "minicolumn." Some investigators equate the vertical string of cell bodies with minicolumns, and others regard a minicolumn as being an entity that extends from the midline of one vertical string of cell bodies through the pale staining interval to the midline of the adjacent string. In this chapter we are taking the stance that the latter definition is correct and in terms of optical density measures taken from digitized images, this means that the size of a microcolumn would be the distance from one density peak, i.e., the axis of a vertical string of cell bodies, to the next density peak. Some examples of the dimensions of minicolumns measured in this way are given in Table 2.

Table 2 Center-to-center spacing of vertical strings of neurons in normal neocortex

Species	Area examined	Center-to-center spacing (μm)	Authors
Monkey	Area 22	36	Buxhoeveden et al. (2001)
Monkey	Areas 46,TL, and TC	22–27	Cruz et al. (2005)
Chimpanzee	Area 22	35–36	Buxhoeveden et al. (2001)
Human	Area 22	50	Buxhoeveden et al. (2001)
Human	Areas 22, 23, and 41	37–47	Seldon (1981)
Human	Medial prefrontal	49–60	Gabbott (2003)
Human	Cingulate cortex	80	Schlaug et al.(1995)
Human	Frontal cortex	51–58	Buxhoeveden et al. (2006)
Human	Visual cortex	34	Buxhoeveden et al. (2006)
Human	Areas S1, 4, 9, and 17	34 average	Casanova et al. (2006)

One of the first investigators to examine the vertical strings of neurons in Nissl stained sections was Seldon (1981), who examined three cytoarchitectonic areas in human auditory cortex, areas TA (area 22), area TB (area 42), and area TC (area 41), using celloidin-embedded material. In these cortical areas the arrangement of the somata of the neurons into vertical strings is very obvious. Seldon (1981) found that in his material the minicolumn width, namely the average width of a string of cells plus the average width of the intervening pale zone, was 36.7–39.7 μm on the right side of the brain and 44.8–46.7 μm on the left side. Consequently, Seldon (1981) suggested that although they are basically organized in similar fashions, auditory cortical areas on the right side of the brain are more diffusely organized than those on the left side.

Among those who have used digitized images to determine the dimensions of minicolumns are Buxhoeveden et al. (2000). Using their method, this group has determined that the minicolumns in area 22 in the superior temporal gyrus of nonhuman and human primates have different dimensions. They conclude that in monkeys, chimpanzees, and orangutans, the minicolumns are 33–36 μm wide, while in area 22 of the human brain the minicolumns are 54 μm wide (Buxhoeveden et al., 2001; 2002a). Other examples of the dimensions of minicolumns revealed by Nissl staining are given in Table 2.

The real point of making measurements of minicolumns from digitized images of Nissl stained material is to determine if there are alterations in the dimensions of the minicolumns between species, in disease states, and in the brains of humans with behavioral disorders.

Alterations in Nissl Stained Minicolumns

There have been a number of studies on the features and dimensions of minicolumns seen in digitized images of vertical Nissl stained sections taken from the brains of patients with a variety of disorders. They will not all be considered here. Consideration will only be given to the differences that have been reported in Alzheimer's disease, in normal aging and in autism, which is the focus of this book.

Alzheimer's Disease

Buldyrev et al. (2000) used a quantitative method derived from condensed matter physics to examine the disposition of neurons in Nissl stained sections from the inferior bank of superior temporal sulcus in normal human brains, in brains from patients with Alzheimer's disease, and in patients with dementia resulting from Lewy body disease. In control brains they find evidence for the presence of minicolumnar ensembles with a periodicity of about 80 μm, but in brains from Alzheimer's patients in whom there is a loss of neurons, they report an almost complete loss of the minicolumnar organization. Further, the relative degree of loss of the minicolumnar organization appears to be directly proportional to the number of neurofibrillary tangles present, but not to the amount of β-amyloid. In brains

from patients with dementia with Lewy bodies, there is a similar disruption of the minicolumnar organization, even though there is little neuronal loss. The authors conclude that this approach is a useful tool for analyzing the anatomical basis for brain disorders.

Normal Aging

Some of the changes in minicolumnar organization seen in Alzheimer's disease will probably due to normal aging, and in area 46 of the rhesus monkey, in which senile plaques are uncommon, Cruz et al. (2004) using a density map method found that there is no age-related reduction in total neuronal density or in microcolumn width, length or periodicity. However, they did found subtle changes that indicate some disorganization of the minicolumns with age.

In a study of human material, Chance et al. (2006) examined minicolumnar spacing and the organization of cells in the cortices of 17 neurologically normal adult humans aged between 40 and 90 years. They examined Nissl stained sections from the planum temporale (area 22), primary auditory cortex (A1), and middle temporal gyrus (area 21) and concluded that compared to individuals under 65, brains from individuals over 65 years of age show a reduction in the width of minicolumns in the medial temporal gyrus and in the planum temporale, but not in primary auditory cortex. This analysis suggests that although there is no extensive loss of neurons in normal aging, the packing density of the neurons in the vertical strings is increased, implying that there is some loss of neuropil with age. This is consistent with reported loss of dendritic branches, dendritic spines, and axon terminal with increasing age (e.g., Peters et al., 2001). Chance et al. (2006) stained some additional cortical sections for Alzheimer-type pathology and determined that although none of their cases met the criteria for diagnosis of Alzheimer's disease, increased plaque load in these normal brains correlates with a decrease in the width of minicolumns.

Autism

Casanova et al. (2002) have compared the brains from autistic individuals with normal brains and found that in Nissl stained vertical sections taken from Brodmann's areas 9, 21, and 22, the minicolumns in the normal and autistic brains have a somewhat different structure. In the autistic brains, the minicolumns are smaller in width, and the neurons within the vertical strings are less tightly packed, leading the authors to conclude that per unit volume of cerebral cortex, the minicolumns are more numerous in the cortices of autistic individuals. Since studies by earlier authors have shown that there is no abnormality in cell density in the cortices of autistic individuals compared to controls (Coleman et al., 1985; Bailey et al., 1993), Casanova et al. (2002) suggest that autism is the consequence of a defect in migration of cells into the cortex during development.

In a later study Buxhoeveden et al. (2006) examined the frontal cortex from the brains of two autistic individuals, one a 3-year-old child and the other a 41-year-old man, and compared the minicolumnar spacing in the dorsal, mesial, and orbital frontal cortices with those of normal brains. They report that in the adult autistic brain the minicolumnar spacing in dorsal frontal cortex is 15% less than in control brains, 23% less in the orbital cortex, and 10% less in the mesial frontal cortex, although the latter difference was not significant. In contrast to the frontal cortex the minicolumn spacing in area 17 was the same in the autistic and control brains. Buxhoeveden et al. (2006) then compared the minicolumnar spacing in the same frontal cortical areas of the 3-year autistic brain with those of controls and found no difference in the spacing in the dorsal and orbital areas, but a statistically smaller spacing in the mesial frontal cortex. For area 17, again there was no difference. The authors suggest that the existence of smaller minicolumns in the frontal cortices of autistic brains means that there is an increase in the number of minicolumns in autistic brains, producing larger than normal frontal lobes in autistic children (see Courchesne, 2004; Carper and Courchesne, 2005). While there might be an increase in the overall numbers of minicolumns in the autistic brain, because the minicolumns are smaller, this would not necessarily lead to an increase in the sizes of the frontal lobes in autistic children.

Essentially these conclusions about autistic brains have been substantiated in a more thorough study of primary sensory (area S1), primary motor (area 4), primary visual (area 17), and frontal association (area 9) cortex using six autistic brains and six age-matched controls (Casanova et al., 2006). Again Casanova and his colleagues find the widths of the strings of neurons to be narrower by 1.5 μm, or 5.5% in the autistic brains, so that the minicolumnar spacing, measured from one density peak to another, is narrower in the autistic brains. The consequence is that the number of minicolumns per linear distance of the cortical sections is greater in autistic brains, even though the number of cells per minicolumn is the same in the autistic and control brains. But because there are more minicolumns in autistic brains, the overall density of neurons is 23% greater than in control brains, although the sizes of the neuronal cell bodies and of their nucleoli are smaller. Since the brains of autistic adults are basically the same size as normal ones, the implication is that the autistic cortex contains more minicolumns that normal brains (see Courschesne and Pierce, 2005). Since there is less neuropil in the autistic brains, this may mean that the dendritic trees, and even the number of synapses per neuron, may be fewer in cortices of the autistic brains.

In a recent review article on the anatomy of autism Amaral et al. (2008) point out that in these studies by Casanova and his colleagues, only 14 cases of autism, 9 of which had seizures and at least 10 with mental retardation, have been examined for minicolumn pathology. Consequently, more studies using a greater number of autistic brains with fewer other complications need to be carried out before any definite conclusions can be reached about changes that can only be attributed to autism. It would also be appropriate to examine brains in which the apical dendritic clusters and myelinated axon bundles have been stained to confirm the sizes of the minicolumns as detected in digitized images from autistic brains.

At present the causes underlying this potential increase in the numbers of minicolumns in autistic brains in unknown, although it must be related to the manner in which neurons are generated to form the minicolumns during development. It is generally assumed that a minicolumn, as the term is used here, is comprised of the neurons that migrate along the same radial glial fiber during development, and in both humans and monkeys the cell divisions that generate the pyramidal cells in the minicolumns occur before embryonic day 40 (Rakic, 1974; 1985). There is evidence that the brains of autistic infants 2–4 months of age are, on average, 10% larger than those of normal infants, and that there is a spurt of growth, and presumably neuron formation, in autistic infants during the first years of life, a spurt that is not present in normal infants. However, after this spurt of growth there is a plateau, so that in adolescents and adults the autistic brain is no larger than the normal brain (see Courchesne et al., 2004; Courchesne and Pierce, 2005). It is suggested that it is during this time of excessive growth in infancy that the minicolumns are laid down and when they would be generated in excess in autistic brains. Casanova et al. (2006) suggest that the reduction in both the sizes of neuronal cell bodies and of the nucleoli in these neurons in the smaller minicolumns could reflect a bias toward shorter connecting fibers in the autistic cortex, since the distances between adjacent neurons are shorter. They further suggest that this bias would favor local computation by neurons, at the expense of the formation of connections between cortical areas and connectivity across the corpus callosum.

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The Developmental Neuropathology of Autism

Thomas L. Kemper

Introduction

There are numerous reviews of the cellular neuropathology of autism (Bauman and Kemper, 1994, 2005; Kemper and Bauman, 1998: Bailey et al., 1998; Palman et al., 2004; Casanova, 2007; Amaral et al., 2008). In this chapter I will discuss the relationship of the various pathologies to developmental events, a subject not covered in detail in these reviews. Emphasis will be placed on the timing and mechanisms of the known pathologies and their possible relationship to the well-documented abnormal pattern of brain growth seen in autistic individuals.

Cellular Neuropathology

The earliest of these developmental abnormalities involve the brain stem. In a unique case, Rodier et al. (1996) reported the nearly complete absence of the superior olive and facial nerve nucleus, with shortening of the brain stem between facial nerve nucleus and the trapezoid body. They concluded that the initiating injury in this autistic brain occurred around the time of neural tube closure, which occurs at about 4 weeks of fetal development (O'Rahilly and Müller, 1994). This timing also corresponds to an increased incidence of autism following exposure to the drug thalidomide during pregnancy (Rodier and Hyman, 1998; Miller et al., 2005).

In an analysis of six autism spectrum disorder (ASD) brains, Bailey et al. (1998) noted additional abnormalities in the brain stem. These included an unusually large arcuate nucleus in one brain and ectopic neurons on the lateral surface of the medulla in four brains. All the nine brains studied by Bauman and Kemper (2005) have shown abnormal superficial clustering of neurons in the inferior olive and in one brain there was an ectopic superficial cluster of neurons adjacent to the inferior

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cerebellar peduncle at the level of the foramen of Luschka. All the ectopic neurons noted in these two reports are derived from a transient germinal zone called the rhombic lip that is present from the 8th to the 20th week of gestation (Sidman and Rakic, 1982). In the cerebellar cortex, it provides neurons for the internal granule cell layer (Sidman and Rakic, 1982; Bayer et al., 1993) and the projection neurons in the deep cerebellar nuclei (Fink et al., 2006). In the brain stem, it provides neurons for the inferior olive, arcuate nucleus, and the basis pontis (Sidman and Rakic, 1982; Bayer et al., 1993). The neurons destined for the inferior olive migrate within the parenchyma of the medulla and those destined for the arcuate nucleus and basis pontis migrate just under its pial surface (Bloch-Gallego et al., 2005). The ectopic neurons on the surface of the medulla described by Bailey et al. (1998) and Bauman and Kemper (2005) are in the position of this subpial migratory stream. The unusually large arcuate nucleus reported by Bailey et al. (1998) is in one of the target areas of this migratory stream. The abnormal superficial lamination of neurons in the inferior olive indicates a defect in the settling in of the migratory neurons within the inferior olive. Also reported by Bailey et al. (1998) are discontinuities and reduplications of the ribbon of the olivary neurons in several cases. These observations were made from single sections of the medulla. In our own material, with serial sections of the brain stem, we noted that these features were equally present in autistic and in control brains (Thevarkunnel et al., 2004). The breaks in the ribbon corresponded to penetrating blood vessels and the reduplication associated with the complex folding pattern of ribbon of neurons in the inferior olive.

In the cerebellar cortex, the most frequently noted pathology is a decreased number of Purkinje cells (Kemper and Bauman, 1998; Palmen et al., 2004; Bauman and Kemper, 2005; Whitney et al., 2008). This pathology is most marked in the posterior lateral part of the cerebellar hemispheres and the adjacent archicerebellar cortex and occurs without evidence of loss of neurons in the inferior olive in the brain stem (Kemper and Bauman, 1998; Bauman and Kemper, 2005). The Purkinje cells have an intimate relationship with the axons of the inferior olivary neurons in the brain stem, such that loss of Purkinje cells at any time after birth leads to loss of neurons in the inferior olive (Holmes and Stewart, 1908; Norman, 1940; Sakai et al., 1994). Since this intimate relationship between the Purkinje cell and the inferior olive is established in the human brain sometime after 29–30 weeks of gestation (Rakic and Sidman, 1970), it is likely that the decrease in number of Purkinje cells occurred before this time. In those brains with a marked decrease in the number of Purkinje cells, there appears to be a concomitant decrease in the number of granule cells (Bauman and Kemper, 2005). The relationship between the number of granule cells and the number of Purkinje cells noted in the autistic brain has been elucidated in rat studies. With prenatal loss of Purkinje cells the number of granule cells is adjusted such that the ratio of Purkinje cells to the number of granule cells is maintained (Chen and Hilman, 1989).

In the cerebral cortex of autistic individuals, the pathology with the earliest time of onset is the evidence for an increased number of unusually small neuronal minicolumns in multiple cortical areas (Casanova et al., 2002; 2006a; 2006b; Casanova, 2007; Buxhoeveden et al., 2006). These minicolumns are the fundamental building

blocks of the cerebral cortex with their origins dating to a time from before the onset of migration of neurons to the cerebral cortex from the germinal ventricular zone (Rakic, 1995). This migration commences at about 6–7 weeks of gestation (Sidman and Rakic, 1982).

Malformations of the cerebral cortex have been frequently noted in the brains of autistic individuals (reviewed by Palmen et al., 2004). In one of the brains reported by Kemper and Bauman (1998) there was a cortical malformation called polymicrogyria that was located in the orbitofrontal region. This malformation has been shown in experimental animals to be the result of aberrant migration of neurons into destructive superficial cortical lesions at a late stage of neuronal migration to the cerebral cortex (Dvorak and Feit, 1977; Dvorak et al., 1978). In the human brain this corresponds approximately to 16–20 weeks of gestation (Sidman and Rakic, 1982; Rakic, 1988). More subtle changes have been frequently reported. Bauman and Kemper (1994) reported discrete areas of indistinct cortical lamination in the anterior cingulate cortex in five of six brains. In other cerebral cortical areas, Bailey et al. (1998) found an increased density of subcortical neurons in four of six brains, in one an increased number of neurons in layer I, and in three disordered cerebral cortical laminar architecture. In the cerebral cortex of eight autistic individuals, Hustler et al. (2006) found in three brains supernumerary neurons in layer I, in six abnormal neuronal clumping and dysplasia, in three disturbed lamination, in three increased number of subcortical neurons, and in three indistinct cortical boundaries.

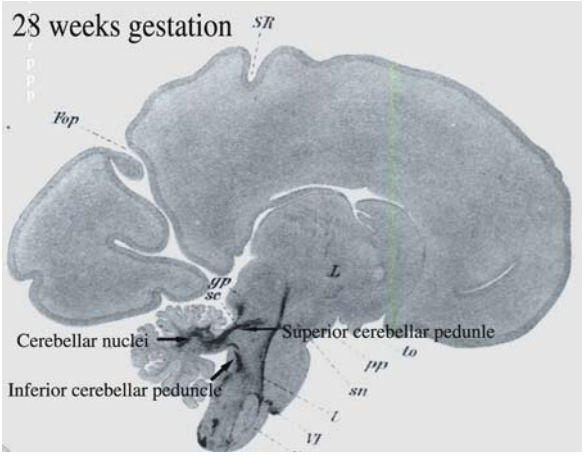
These more subtle malformations within the cerebral cortex and subcortical white matter appear to be due to two different pathological processes. One of these processes is an abnormality in neuronal migration. In normal development, migratory neurons from the germinal ventricular epithelium first appear in the cerebral cortex at about 6–7 weeks of gestation with the migration continuing until about 16–20 weeks of gestation (Sidman and Rakic, 1982; Rakic, 1988). Rakic (1988) has divided abnormalities in the migration of these neurons into three broad categories: complete failure of migration, detention of migratory neurons along their migratory pathway, and aberrant placement (settling in) of post-mitotic neurons within their target structure. In the autistic brains, the disordered lamination and abnormal distribution of neurons within the cerebral cortex are examples of the latter category. The increased number of neurons in the white matter could be accounted for by an arrest of neurons in this location during their migration to the cerebral cortex. However, the subcortical neurons associated with an arrest in migration generally occur as clusters of cells (Harding and Copp, 1997) rather than the scattered individual neurons noted in the autistic brains. Another possible mechanism for the persistence of neurons in the subcortical white matter is the failure of resolution of a transient neuronal zone called the primordial plexiform layer or preplate. In the earliest stages of cerebral cortical development, the definitive neurons of the cerebral cortex migrate into the primordial plexiform layer, separating it into the future layer I and scattered neurons deep into the definitive cerebral cortex, referred to as the subplate (Marin-Padilla, 1988; Super et al., 1998). The subplate zone is unusually prominent in the human brain, where it reaches its peak development in the 24th week of gestation and is largely gone by the 6th postnatal week (Kostovic and

Rakic, 1990). It has been shown to play an essential, transient role in the development of definitive cerebral cortical circuits (Shatz et al., 1998; Super et al., 1998; Okhotin and Kalinichenko, 2003). The presence of both an excess number of neurons in layer I and in the subcortical white matter in the autistic brain suggests a lack of proper resolution of this transient zone.

In the limbic system, Bauman and Kemper (1994, 2005) and Kemper and Bauman (1998) have reported that the neurons appear unusually small and more tightly packed than in age- and sex-matched controls. This was noted in the hippocampal fields CA1-4, subiculum, entorhinal cortex, mammillary body, amygdala, and medial septal nucleus. This pattern of change is reminiscent of an early stage of development and suggests a curtailment of maturation. Analysis of the dendritic tree with the Golgi method for staining individual neurons has also shown evidence of this curtailment in hippocampal fields CA1 and CA4 (Raymond et al., 1996). These observations have been challenged by two recent studies. Bailey et al. (1998) reported, from a single section of the hippocampus, increased cell-packing density in the hippocampus in only one of five autistic cases. Using modern stereology, Schumann and Amaral (2006) were unable to confirm the presence of an increased neuronal packing density and decreased neuronal size in the amygdala of nine autistic individuals. A possible difference between their cases and those reported by Bauman and Kemper (1994, 2005) and Kemper and Bauman (1998) is that their cases were all seizure free, a frequent occurrence in the earlier reported cases. Further studies in these limbic areas, using modern stereological techniques, will be needed to resolve these apparent discrepant observations.

Another neuropathology noted in the autistic brain is abnormalities in neuronal size and evidence of postnatal neuronal loss (reviewed by Kemper and Bauman, 1998; Bauman and Kemper, 2005). This has been found in the deep cerebellar nuclei and in the inferior olive, nuclei that are topographically related to cerebellar cortex, and in the nucleus of the diagonal band of Broca (NDB) in the septum. In all of the childhood ASD brains (ages 5–13 years), these neurons were consistently enlarged and appeared to be present in adequate numbers. In contrast, in the older brains, the neurons in the fastigial, globose, and emboliform nuclei of the deep cerebellar nuclei and in the NDB were observed to be small and pale and reduced in number. In older individuals, the neurons in the dentate nucleus appeared comparable in size to age-matched controls and the neurons in the inferior olive reduced in size and pale. Neuronal swelling and neuronal swelling, followed by atrophy and cell loss is an unusual neuropathology. Enlarged neurons similar to those noted in these autistic brains have been reported in a variety of circumstances associated with altered connectivity in nuclei that are closely related to the cerebellar cortex. These enlarged neurons have been most frequently reported in the inferior olive following destructive lesions in the central tegmental tract, a tract with a heavy projection to the inferior olive as well as with destructive lesions that involve the dentate nucleus, superior cerebellar peduncle, and red nucleus. This interrelated circuitry is often collectively referred to as the Guillain–Mollaret triangle (Gautier and Blackwood, 1961; Goto and Kaneko, 1981). In the human brain, olivary neuronal hypertrophy is first noted 3 weeks following the lesion with evidence of neuronal loss noted

91/2 months later (Goto and Kaneko, 1981). Abnormally enlarged neurons have also been reported in the human dentate nucleus. Fukutani et al. (1996) reported this finding in one of three cases of sporadic olivopontocerebellar atrophy, a disease associated with marked loss of neurons in the inferior olive, basis pontis, and cerebellar cortex. In this brain it occurred without evidence of neuronal cell loss in the dentate nucleus. Arai et al. (1988) reported enlarged dentate neurons in the human brain in association with loss of Purkinje cell innervation. What these various reports in the literature seem to suggest is that enlarged neurons in the cerebellar circuitry may represent a reaction to a variety of perturbations, a peculiarity that occurs primarily in response to altered connectivity. Only the enlarged neurons in the dentate nucleus in the 5–13-year-old autistic individuals can be understood in terms the circumstances noted by Arai et al. (1988). These could be related to the decreased density of Purkinje cells in the ASD brains with a concomitant loss of their axonal projection to the dentate nucleus. The enlarged neurons in the inferior olive are unlikely to be related to the abnormalities noted in the dentate nucleus. There is no evidence of a destructive lesion in the dentate nucleus, and if one were present the olivary neuronal hypertrophy would not persist for years as it does in the autistic brain. The close topographic relationship of the olivary neuronal hypertrophy followed by atrophy to the cerebellar cortical areas with the most marked decrease in density of Purkinje cells suggests that these are related. None of the circumstances that have been reported in the literature to be associated with neuronal hypertrophy could account for the hypertrophy of the neurons in the globose, emboliform, and fastigial cerebellar nuclei or the neurons in the nucleus of the diagonal band of Broca (NDBB). Elsewhere (Kemper and Bauman, 1993), we had suggested another mechanism that could account for this peculiar pattern of hypertrophy followed by atrophy of the neurons in these tightly interrelated nuclei in the cerebellar circuitry. As mentioned above, the failure of the occurrence of retrograde loss of inferior olivary neurons in association with a marked decrease in the number of Purkinje cells in the autistic brain is consistent with a loss of Purkinje cells that occurs before 29–30 weeks of gestation. Just before this time, at 28 weeks of gestation, it can be seen in the illustrations of the studies of the development of myelination in the human brain by Flechsig (1920), that the olivo-cerebellar fibers, the deep cerebellar nuclei, and the superior cerebellar peduncle show advanced myelination at that time, with no indication of myelination of the white matter of the cerebellar hemispheres (Fig. 1). Further, Rakic and Sidman (1970), in their seminal study of the prenatal development of the human cerebellum, noted that at 26 weeks of gestation the cerebellar nuclei are "virtually mature," and Marin-Padilla (1985) reports that the olivary climbing fibers first arrive at the human Purkinje cell layer at 28 weeks of gestation. These observations, therefore, suggest that a well-developed fetal circuit is present at about 28 weeks of human gestation that does not appear to involve the cerebellar cortex. Myelination of the afferent and efferent axons related to the cerebellar cortex and the innervation of the Purkinje cells by climbing fibers appear at a later age. In the adult brain, the projection from the inferior olive to the cerebellar nuclei is represented by a thin collateral of the robust olivary projection to the Purkinje cells (Sugihara and Shinoda, 1999). We therefore postulated that the early



decrease in the number of Purkinje cells might have favored the persistence of a fetal circuit and that the persistence of this abnormal connectivity could account for the initially unusually large neurons in cerebellar nuclei and perhaps their later atrophy and neuronal loss due to instability of this persistent circuit. A similar mechanism might also account for the unusually large neurons in the nucleus of the diagonal band of Broca as it projects to an abnormal neuronal substrate in the hippocampal complex and the amygdala (Bauman and Kemper, 1994).

Abnormal Postnatal Brain Growth

An unusually large head in individuals with autism, first noted by Kanner in 1943, has been repeatedly documented, with about 20% of ASD subjects having head circumferences greater than the 97 percentile (Fombonne et al., 1999). Hobbs et al. (2007), in second trimester ultrasounds of 45 fetuses later diagnosed as autistic, found that body size and head circumference were normal for gestational age. In a meta-analysis of the literature on postnatal brain growth, Redcay and Courchesne (2005) found data on head circumference, autopsy brain weight, and brain volume determined by MRI for 531 autistic individuals. In these studies brain size was reliably estimated by measurements of head circumference for neonates, infants, and young children and then by MRI and autopsy brain weights as head circumference becomes less reliable in older individuals. Fitted curves for head circumference and brain volume from 15 of these studies revealed a largely consistent pattern of age-related changes in brain size. Brain size was slightly reduced at birth, dramatically increased in size in the first postnatal year with then a plateau in brain growth, with the majority of autistic individuals with a normal brain size by adulthood. There were thus two identified abnormalities in brain growth, an early, abnormally rapid rate of brain growth followed by an abnormally slowed rate of brain growth.

Several later studies have explored the timing of the abnormal postnatal brain growth during the first postnatal year. Hazlett et al. (2007) reported the head circumference of the autistic individuals began to diverge at 12 months of age with the rate of head circumference growth then continuing to increase throughout the study period (up to 35 months). Dawson et al. (2007) calculated the rate of increase from birth to 36 months. They concluded that the abnormal increased rate of head growth in head circumference was confined to the first 12 postnatal months. In a later paper from this same group (Webb et al., 2007), they stratified the data on head circumference into seven age groups noting that the significant increase occurred after 6 months of age. In contrast, Dementieva et al. (2005) reported that the increase in head size begins shortly after birth. They obtained physician-recorded sequential head circumference measurements on 251 individual with well-documented autism. Fifteen of these had sufficient data to determine the rate of increase in head circumference growth from 0 to 1 month, from 2 to 6 months, and from 6 to 12 months. They found that the overgrowth was not present at birth, but that there was a "sudden and excessive" increase in head size between 1 and 2 months.

There is some data on the timing of the deceleration of brain growth and the time at which brain size becomes comparable to age-matched controls. In the study of Dawson et al. (2007), the rate of brain growth in ASD infants from birth to 36 months became comparable to controls from 12 to 36 months of age. According to Courchesne et al. (2003), maximum brain size occurs at 4–5 years and then becomes comparable to controls by adolescence and adulthood. Courchesne and Pierce (2005), in their review the literature on head size in ASD, noted that the maximum difference in brain size was at 2–4 years, followed by a decline in difference in later childhood. Redcay and Courchesne (2005), in their meta-analysis found an "abrupt cessation" in brain growth by 2–4 years followed by a plateau, with the age group, 2–5 years with the greatest difference in brain size. Lainhart et al. (2006) suggested that the occurrence of macrocephaly reached its peak at 3–5 years of age and then remained stable. Aylward et al. (2002), in a MRI study of volume of 67 individuals ages 8–18, found an increased volume only in those less than 12 years of age.

Lainhart et al. (2006) pooled consistently recorded data on head circumferences from 338 well-documented individual with autism spectrum disorder, 2–49 years of age, from 10 centers in the NIH Collaborative Program of Excellence in Autism. In the entire group 17% were macrocephalic, with 12–20% macrocephalic by 3–5 years, a rate that then remained stable. They found that head circumference of the autistic individuals showed a normally distributed curve, with a shift to the right, suggesting that an increase in head circumference may be present in all autistic individuals. They noted that three other studies had not found a normally distributed head circumference (Lainhart et al., 1997; Fombonne et al., 1999; Miles et al., 2000). These three studies, however, included a smaller number of subjects, had more individuals with mental retardation, and included subjects that did not have idiopathic autism.

Several studies that have examined the expected relationship between body length (height) and head size in autistic individuals and have failed to find evidence

for this relationship (Lainhart et al., 1997, 2006; Miles et al., 2000; Courchesne et al., 2003; Hazlett et al., 2007; Dawson et al., 2007). Head size in ASD does appear to be related to parental head size with a striking incidence of macrocephaly in either parent (Stevenson et al., 1997; Miles et al., 2000; Lainhart et al., 2006). This occurs with both normocephalic and macrocephalic autistic individuals. In the study of Miles et al. (2000), the incidence of macrocephaly was 45% in parents of macrocephalic autistic individuals and 37% with normocephalic autistic individuals. This strikingly high incidence of increased head circumference in the first degree relatives suggests that hereditary factors may play a role.

Relationship Between Cellular Pathology and Abnormal Brain Growth

The pathological changes noted above as well as later postnatal developmental processes could potentially account for the well-documented abnormalities in postnatal brain growth. Ideally, such a mechanism should account for the early, accelerated increase in brain growth, and its later abnormally slowed rate of growth. As noted above, the early postnatal increase in brain size is often striking, with the size of many brains two standard deviations above normal. The sheer size of this increase dictates that the major player is the forebrain. Within the forebrain, the volume of myelinated fibers is likely to be responsible as there is a marked increase in myelination of subcortical axons in the first few postnatal years (Yakovlev and Lecours (1967). In agreement with this, Amaral et al. (2008), in their review of the literature, note this enlargement of the white matter in ASD is particularly evident in the younger individuals. In the autistic brain, this increase in volume of myelinated fibers is predominantly in the superficial, subcortical myelinated fibers, fibers that are predominantly involved in corticocortical projections (Herbert et al., 2004).

Possible interrelationships between brain pathology and abnormal postnatal brain growth include abnormalities in the normal elimination of neurons, of synapses, or of myelination fibers as well as prenatally determined pathology such as aberrant connectivity attendant to increased number of minicolumns, aberrant connectivity-associated cerebrocortical malformations, persistent fetal circuits, and abnormalities in growth factors such as neuropeptides. The possible role of neuronal elimination and structural and biochemical abnormalities in myelin has not been explored.

Failure of cerebral cortical synaptic elimination is an unlikely possibility based on the timing of this phenomenon in normally developing individuals. In these studies (Huttenlocker et al., 1979, 1982, 1997), the primary visual and auditory cortices have peak synaptic density at 8–12 months and 3 months, respectively, with the period of synaptic elimination extending up to 11–12 years of age. In the frontal association cortices peak synaptic density occurs later at 1–2 years or at 3 1/2 years, depending on location, with the period of synaptic elimination extending to 12–15 years. Thus, the period of most active brain growth corresponds most closely with

the normal growth of synaptic density, not to the later, more prolonged period of synaptic elimination.

Developmental elimination of axons and myelinated fibers in the forebrain has been well studied in the macaque monkey in the corpus callosum (LaMantia and Rakic, 1990) and the anterior commissure (LaMantia and Rakic, 1994). In both commissures the peak number of axons occurs at birth (embryonic day 165), with then rapid axonal elimination and the establishment of the adult number of axons achieved by 3rd to the 5th postnatal month. The eliminated fibers are small, unmyelinated axons. Myelination in these brains begins at birth in the macaque and continues until 1 year of age. Comparable information in the human brain to that provided by these primate studies is available only for myelination. According to Yakovlev and Lecours (1967), myelination of anterior commissures and corpus callosum begins during the 4th postnatal month and then extends to beyond the first decade. In this study the superficial, subcortical white matter in the normally developing brain also begins myelination at the same time as the commissures, but its timetable is more protracted. This would place the beginning of the period of axonal elimination in these locations to about the 4th month, which is co-incident with the timing of the increased growth in size of the autistic brain.

Another possible etiology for the accelerated postnatal brain growth in the autistic brain is an increase in the number of unusually small minicolumns in the cerebral cortex reported by Casanova et al. (2002, 2006a, 2006, 2007) and Buxhoeveden et al. (2006). Each minicolumn has its own attendant connectivity with each other and with other parts of the brain. An absolute increase in their number could increase the number of connecting fibers and the bulk of these fibers could account for the megalencephaly. The key piece of information in these studies is the measurement of the size, width, and density of these minicolumns in different cortical areas. The absolute number of minicolumns would depend on measurement of the surface extent of the individual cortical areas or of the entire cerebral cortex. With a more extensive surface, or even one comparable to controls, there would be an increased number of the smaller minicolumns. This measurement of surface extent is not available for the autistic brain. Of particular interest is the demonstration that this phenomenon is not present in all cortical areas, with a predilection shown for the prefrontal areas and the anterior cingulate cortex (Casanova, 2006b; Buxhoeveden et al., 2006). However, in Casanova et al. (2002) only one prefrontal region (BA 10) showed a significant decrease in minicolumn size. Two other prefrontal areas (BA 9 and 11) failed to show a significant difference. The very selective nature of this change in the autistic brain may be insufficient to account for the robust postnatal increase in head size.

Another possibility is that the frequently noted cerebrocortical malformations in the autistic brain may be associated with aberrant, increased connectivity. Evidence for this comes from studies of focal cerebrocortical malformations in experimental animal models. Rosen et al. (2000) explored the connectivity of experimentally induced polymicrogyria and showed that there was an enhancement of ipsilateral cerebrocortical projections, projections to cortical regions not normally innervated in the adult brain, and a decreased callosal connectivity. Jenner et al. (2000) studied

the abnormal focal accumulations of neurons in layer I that are found in the New Zealand black mouse. They found that cortical connectivity of these lesions was more "intense" than expected, but with a similar distribution pattern to that found following injections in comparable, unaffected areas. As with the lesion studied by Rosen et al. (2000), there were virtually no callosal projections.

Possible evidence for a persistent fetal circuit is provided by the abnormal presence of an increased number of neurons in cerebrocortical layer I and in the subcortical white matter that has been noted in several autistic brains. These neurons have been shown to normally play an essential, transient role in the development of definitive cerebral cortical circuits, particularly in relationship to thalamocortical connections (Shatz et al., 1998; Super et al., 1998; Okhotin and Kalinichenko, 2003). In this regard, it is of interest to note that Kleinhans et al. (2008), in an fMRI functional study of face recognition, noted in ASD a failure of the significant activation of the thalamus that was noted in the controls.

The best evidence for abnormal fetal circuits is found in the cerebellum, as discussed in the section on cellular pathology. Because of its relatively small size, it is unlikely to contribute to the increased postnatal brain growth.

Nelson and Bauman (2003) measured numerous neuropeptides in archived neonatal blood of children later diagnosed with autism, mental retardation, cerebral palsy, and controls. They found, in comparison with controls, significant elevations in concentrations of vasointestinal peptide (VIP), calcitonin gene-related peptide (CGRP), brain-derived neurotrophic factor (BDNF), and nerve growth factors (NT4/5) in children with autism and in those with mental retardation without autism. There was no measure that distinguished autism from mental retardation.

Concluding Remarks on Abnormal Grain Growth

The diverse pathological findings that are present in subsets of the brains of autistic individuals suggest that an increase in brain size could be an outcome of several different prenatal pathological processes. As can be seen from the above discussion, it is uncertain if any of the observed pathologies are etiologically related to the abnormal brain growth in autistic individuals. One possibility to explore is the effect of as yet an unspecified genetic growth factor. In support of this is the incidence of macrocephaly in 45% of parents of macrocephalic autistic individual and of 37% of parents with normocephalic autistic individuals (Miles et al., 2000).

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Understanding Alterations During Human Brain Development with Molecular Imaging: Role in Determining Serotonin and GABA Mechanisms in Autism

Diane C. Chugani

Brain development involves a series of overlapping processes, occurring with distinct time courses, during which brain structure and function is established. During this process, there are highly dynamic changes in many neurotransmitter systems. Changes in these neurotransmitter systems during brain development, including the serotonergic and GABAergic systems, are proposed as contributing to the pathophysiology of autism. Positron emission tomography (PET) measures of neurochemical processes can be used to delineate normal biochemical patterns of brain maturation. The identification of deviations in the development of biochemical processes in autism can provide a rationale for a time limited pharmacological or behavioral intervention to impact that particular process.

Studies of Brain Development with PET

At present, the time courses for relatively few molecular processes have been studied in humans in vivo. PET studies in humans have shown significant changes in glucose metabolism during normal brain development (Chugani et al., 1987). Similarly, PET studies of serotonin synthesis (Chugani et al., 1999) and $GABA_A$ receptor binding (Chugani et al., 2001) have also demonstrated significant changes during human brain development.

Serotonin Synthesis

Ontogeny studies in nonhuman primates demonstrate changes in neurotransmitter content and receptor binding (Goldman-Rakic and Brown, 1982; Lidow et al., 1991). For example, in the macaque, there is a steep rise in cortical serotonin

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content beginning before birth and reaching a peak at 2 months of age, followed by a slow decline until about 3 years of age, when puberty occurs (Goldman-Rakic and Brown, 1982). The same group of investigators has reported a similar time course for expression of serotonin receptors (Lidow et al., 1991). Human whole brain serotonin synthesis capacity measured at different ages using the tryptophan analogue alpha[^{11}C]methyl-L-tryptophan (AMT) and PET showed changes with development as well. In nonautistic children, serotonin synthesis capacity was >200% of adult values until the age of 5 years and then declined toward adult values (Chugani et al., 1999). Serotonin synthesis capacity values declined at an earlier age in girls than in boys. These data suggest that humans undergo a period of high brain serotonin synthesis capacity during childhood followed by a decline toward adult values and that there is gender differences.

GABA$_A$ Receptors

An understanding of human GABA$_A$ receptor ontogeny is highly relevant in elucidating the pathophysiology of neurodevelopmental disorders in which GABAergic mechanisms play a role, as well as understanding age-related differences in the pharmacology of drugs acting on this system. Age-related changes in the brain distribution of the GABA$_A$ receptor complex was measured in vivo using PET in children with epilepsy under evaluation for surgical treatment (Chugani et al., 2001). PET imaging was performed using the tracer [^{11}C]flumazenil (FMZ), a ligand which binds to alpha subunits of the GABA$_A$ receptor. FMZ binding was quantified using a two-compartment model yielding values for the volume of distribution of the tracer in tissue. All brain regions studied showed the highest value for [^{11}C]flumazenil volume of distribution at the youngest age measured (2 years), and the values then decreased exponentially with age. Medial temporal lobe structures, primary visual cortex, and thalamus showed larger differences between 2 years of age and adults (approximately 50% decrease) as compared to basal ganglia, cerebellum, and other cortical regions (which showed 25–40% decreases from 2 years to adulthood). Furthermore, subcortical regions reached adult values earlier (14–17.5 years) compared to cortical regions (18–22 years). Like serotonin, GABA$_A$ receptor binding differed with age, although the time course of changes with age are distinctly different from those for serotonin.

Alteration of Serotonin Synthesis Capacity in Autism

In order to determine whether there are brain serotonergic abnormalities in children with autism, serotonin synthesis capacity was measured in vivo with PET, using alpha[^{11}C]methyl-L-tryptophan as the tracer. Two different types of serotonergic abnormalities were measured in children with autism (Chugani et al., 1997, 1999; Chandana et al., 2005). The first is a difference in whole brain serotonin

synthesis capacity in autistic children compared to age-matched nonautistic children. As described above, serotonin synthesis capacity was >200% of adult values until the age of 5 years and then declined toward adult values in nonautistic children. In contrast, serotonin synthesis capacity in autistic children increased gradually between the ages of 2 years and 15 years to values 1.5 times the adult normal values (Chugani et al., 1999). These data suggested that humans undergo a period of high brain serotonin synthesis capacity during early childhood, and that this developmental process is disrupted in autistic children. The second type of abnormality reported relates to focal abnormalities in brain serotonin synthesis. Asymmetries of AMT uptake in frontal cortex, thalamus, and cerebellum were visualized in children with autism (Chugani et al., 1997). In addition, we measured brain serotonin synthesis in a large group of autistic children and related these data to handedness and language function (Chandana et al., 2005). Cortical AMT uptake abnormalities were objectively derived from small homotopic cortical regions using a predefined cutoff asymmetry threshold (>2 SD of normal asymmetry). Autistic children demonstrated several patterns of abnormal cortical involvement, including right cortical, left cortical, and absence of abnormal asymmetry. Groups of autistic children defined by the presence or absence and side of cortical asymmetry, differed on a measure of language as well as handedness. Autistic children with left cortical AMT decreases showed a higher prevalence of severe language impairment, whereas those with right cortical decreases showed a higher prevalence of left and mixed handedness. These results suggest that global as well as focal abnormally asymmetric development in the serotonergic system could lead to miswiring of the neural circuits specifying hemispheric specialization.

Alterations of $GABA_A$ Receptor Binding in Autism

Quantification of $GABA_A$ receptor distribution in the brain, using [^{11}C]flumazenil PET can be accomplished by calculating the volume of distribution of the tracer in the brain tissue. The macroparameter volume of distribution characterizes both receptor density and affinity of $GABA_A$ receptors and allows comparison across patient groups. The volume of distribution for nine autistic children with autism were compared to the developmental curve established for nonautistic children with epilepsy described above (Chugani et al., 2002). Four children with autism showed whole brain volume of distribution values which were below the curve for nonautistic children (mean $\pm$ SD $=$ 3.53 ml/g $\pm$ 0.18), while the remaining five autistic children (mean $\pm$ SD $=$ 4.78 ml/g $\pm$ 0.38) had values fell within the range of nonautistic children of the same age. These preliminary studies show that a subset of children with autism show decreased $GABA_A$ receptors, while in others this receptor does not appear to be affected. Four children with chromosome 15 duplications were also studied (unpublished data). All four of these children showed lower values than the nonautistic group of children. Molecular imaging may be useful for determining whether a particular neurotransmitter system is involved in a particular child, given the heterogeneity of etiology in children diagnosed with autism.

Pharmacological Intervention in Autism to Restore Neurochemical Developmental Patterns

Pharmacological interventions in autism have been carried out predominantly in older children and adults (for reviews see, Posey and McDougle, 2001; Palmero and Curatolo, 2004). The primary goal of these studies was to target certain disruptive behaviors such as aggression, self-injurious behavior and sleep problems, or specific behaviors characteristic of autism, such as stereotyped behaviors (reviewed in, Aman, 2004; Bostic and King, 2005). Pharmacological intervention to restore the normal pattern of brain biochemical development based on altered patterns through molecular imaging, such as treatments with agents impacting serotonin or GABA neurotransmission, represents a new direction for the pharmacological treatment of autism.

Mechanisms of Serotonin Effects on Brain Development

Evidence from both pharmacological and gene knockout experiments (for review see Gaspar et al., 2003) demonstrates that serotonin plays a role in modulation of synaptogenesis. Immunocytochemistry for serotonin and [^{3}H]citalopram binding to serotonin uptake sites both have demonstrated a transient serotonergic innervation of primary sensory cortex between postnatal days 2 and 14 during the period of synaptogenesis in rat cortex (D'Amato et al., 1987; Cases et al., 1995). Two early studies (Bennett-Clarke et al, 1996; Lebrand et al., 1996) reported that this transient innervation actually represented transient expression of the high-affinity serotonin transporter and vesicular monoamine transporter by glutamatergic thalamocortical neurons. The serotonin transporter is transiently expressed by glutamatergic thalamocortical afferents (Bennett-Clarke et al., 1996; Lebrand et al., 1996) during the first 2 postnatal weeks in rats. During this period, these thalamocortical neurons take up and store serotonin, although they do not synthesize serotonin. While the role of serotonin in glutamatergic neurons whose cell bodies are located in sensory nuclei of the thalamus is not yet known, there is evidence that the serotonin concentration must be neither too high nor too low during this period. Thus, depletion of serotonin delays the development of the barrel fields of the rat somatosensory cortex (Blue et al., 1991; Osterheld-Haas and Hornung, 1996) and decreases the size of the barrel fields (Bennett-Clarke et al., 1994). Conversely, increased serotonin during this critical period, as in the MAO-A knockout mouse, results in increased tangential arborization of these axons, resulting in blurring of the boundaries of the cortical barrels (Cases et al., 1996). Decreased or increased brain serotonin during this period of development results in disruption of synaptic connectivity in sensory cortices (Cases et al., 1995; Bennett-Clarke et al., 1994; Cases et al., 1996). Furthermore, disruption of serotonin transporter functions impairs the cerebral glucose metabolism response to whisker stimulation (Esaki et al., 2005). The effect of serotonin on synaptogenesis and synaptic function is not limited to the

sensory cortices. For example, Yan et al. (1997) have reported that depletion of serotonin with PCA or 5, 7-dihydroxytryptamine in neonatal rat pups resulted in large decreases in the numbers of dendritic spines in hippocampus.

The changes in serotonin receptor density, serotonergic innervation, and serotonin synthesis with age suggest that serotonin plays an important role in brain development. Indeed, there is a body of evidence indicating that serotonin regulates several aspects of brain development, including regulation of cell division, differentiation, neurite outgrowth, and synaptogenesis. These effects have been observed on serotonergic neurons as well as in the tissues innervated by serotonergic terminals. There are several different mechanisms by which serotonin influences brain development. These include regulation of trophic factors and direct regulation of activity-dependent plasticity. There is evidence that one mechanism by which serotonin exerts trophic effects during brain development may be through the regulation of trophic factors such as $5HT_{1A}$-mediated release of S100b (Nishi et al., 1996) and brain-derived neurotrophic factor (BDNF) (Galter and Unsicker, 2000). One study has reported evidence that serotonin, the $5HT_{1A}$ receptor, BDNF, and its receptor trkB form an autoparacrine loop which regulates the differentiation of serotonergic neurons (Galter and Unsicker, 2000). This study reported that serotonin and the $5HT_{1A}$ agonists BP-554 and 8-OH-DPAT (but not $5HT_{1B}$ and $5HT_{1D}$ agonists) increased the numbers of cultured raphe neurons expressing serotonergic markers and BDNF rRNA. Treatment with the $5HT_{1A}$ antagonist WAY-100635 or trkB-IgG fusion protein blocked the induction of serotonergic markers in the cultures.

During brain development, activity-dependent processes are also thought to play a role in the refinement of synaptic connections. Axons are thought to compete for post-synaptic targets during critical periods when synapses are stabilized or lost depending on synaptic activity (Goodman and Shatz, 1993). One mechanism by which synapses are believed to be stabilized is by long-term potentiation (LTP). Interestingly, serotonin and its alterations during development have effects on LTP. These effects of serotonin on LTP have been documented in several brain regions, including somatosensory cortex (Isaac et al., 1997), visual cortex (Edagawa et al., 1998a, b, 1999, 2001; Kojic et al., 2000), spinal cord (Li and Zhuo, 1998), and hippocampus (Tecott et al, 1998). In addition, evidence for serotonergic modulation of synaptic development has been demonstrated for the lateral superior olive in developing gerbils (Fitzgerald and Sanes, 1999) and for segregation of retinal projections in MAO-A knockout mice (Upton et al., 1999). In summary, it is now clear that serotonin influences postnatal synaptogenesis in multiple brain regions.

Mechanisms of GABA Effects on Brain Development

In the mature brain, GABA functions as an inhibitory neurotransmitter, but changes, in the developing brain, in the distribution of expression of $GABA_A$ receptor subunits indicate that it may function as a neurotrophic factor affecting neural differentiation, growth, and circuit organization. The density of $GABA_A$ receptors measured by both [^{3}H]muscimol and [^{3}H]flunitrazepam binding in rhesus monkey

cortex increases after birth to reach a maximum at 2–4 months of age (Lidow et al., 1991). Values at the maximum were close to twofold higher than adult values, and then gradually declined to adult values at 3 years of age, which is the age of puberty in the rhesus monkey. Studies of the developmental profile of cortical and subcortical $GABA_A$ receptor subunits in marmoset monkeys (Hornung and Fritschy, 1996) and macaque monkeys (Kultas-Ilinsky et al., 1998; Huntsman et al., 1999) demonstrate dramatic regional and laminar-specific changes which suggest that distinct receptor subtypes are required at various stages of brain maturation. There is evidence that changes in subunit composition are related to critical periods of synaptic plasticity in developing visual (Huntsman et al., 1994; Huang et al., 1999) and somatosensory cortices (Golshani et al., 1997; Huntsman et al., 1995).

There are a number of pathology studies in postmortem human brain showing alteration in GABA synthetic enzymes and receptors in autism. Reduced protein levels of GAD65 and GAD67, two enzymes which are rate limiting in the conversion of glutamate to GABA, were found in the parietal and cerebellar cortices of autistic brains (Fatemi et al., 2002). Decreased GAD65 mRNA levels were found in a subpopulation of neurons in the cerebellar dentate nuclei and decreased GAD67 mRNA in cerebellar Purkinje cells in autistic subjects (Yip et al., 2007; 2009). Decreased binding of [^{3}H]flunitrazepam to the $GABA_A$ receptor was reported in hippocampus (Guptill et al, 2007), and $GABA_A$ receptor subunit expression was found to be decreased in frontal and parietal cortices and cerebellum, as well as in cerebellum (Fatemi et al., 2009). These pathology studies are consistent with the PET imaging studies showing whole brain decreases in [^{11}C]flumazenil binding in a subset of children with autism.

Cytogenetic studies show abnormalities in chromosome 15 in autism, specifically 15q11-q13, the region coding for several proteins including $GABA_A$ receptor subunit genes (*GABRB3, GABRA5*, and *GABRG3*) (Silva et al, 2002, Menold et al., 2001, Buxbaum et al, 2002). Moreover, symptoms of autism can be associated with both Prader–Willi and Angelman syndromes, both of which involve alterations in the 15q11-q13 region. Although autism and Angelman syndromes are distinct disorders, there is considerable overlap among them, for conditions characterized by language deficit, seizures, mental impairment, behavior abnormalities, and sleep disturbances. The region 15q11-q13 contains the disease loci for Angelman syndrome (*UBE3A*) resulting from deletion or mutation within maternal chromosome 15q11-q13. Other potential candidate genes in this region include a cluster of three $GABA_A$ receptor subunits ($\beta3$, $\alpha5$, and $\gamma3$), and the *GABRB3* gene, which codes for $\beta3$ subunit, is deleted in most patients with Angelman syndrome. Based on investigations of knockout mouse (Homanics et al., 1997, DeLorey et al., 1998; DeLorey et al., 2008), it was suggested that the lack of *GABRB3* gene could contribute to the most of the clinical symptoms in Angelman syndrome. Interestingly, Angelman patients with a maternal deletion of 15q11-q13 leading to the loss of $\beta3$ subunit showed significantly decreased binding of [^{11}C]flumazenil in the frontal, parietal, hippocampal, and cerebellar regions in a PET study (Holopainen et al., 2001). These findings were reproduced in animal models of Angelman syndrome (Sinkkonen et al., 2003). If these observations are correct,

altered GABAergic mechanisms may have a critical role in the clinical manifestation of several neurological disorders with similarities in phenotype to Angelman syndrome, in particular autism. The same region (q11-q13) on chromosome 15 was implicated in autism based on several observations showing chromosomal duplications in autistic individuals and evidence of linkage and linkage disequilibrium in autistic families (Cook et al., 1997, Bass et al., 2000). Specifically, linkage disequilibrium was identified in *GABRB3* and *GABRA5*, as well as at the Angelman syndrome gene *UBE3A* in autism families (Cook et al., 1998, Martin et al., 2000).

Combining Pharmacological and Behavioral Interventions

The combination of particular behavioral interventions during critical period targeted pharmacotherapy of serotonin or GABA neurotransmission should be considered. For example, it is well demonstrated that GABA mechanisms are involved in the refinement of ocular dominance columns. Treatment with GABAergic drugs during a critical period alters the time course of this development (Hensch et al., 1998). There is abundant evidence that developmental changes in GABA neurotransmission are related to critical periods of activity-dependent synaptic plasticity in response to sensory experience in animals (Wolf et al., 1986; Ramoa et al., 1988; Reiter and Stryker, 1988). Furthermore, there are dramatic changes in $GABA_A$ receptor subunit composition in developing brain, for example, in visual cortex (Huntsman et al., 1994; Huang et al., 1999), somatosensory cortex (Golshani et al., 1997; Huntsman et al., 1995), and cerebellum (Carlson et al, 1998). A specific role for benzodiazepine-sensitive $GABA_A$ receptors in the critical period for establishing ocular dominance has been suggested by investigators, who have demonstrated that infusion of diazepam can restore visual cortex plasticity in mice with gene-targeted disruption of GAD65 (Hensch et al., 1998). GAD65 knockout mice showed no shift in ocular dominance with eye closure during the critical period. However, ocular dominance shift in response to eye closure *did* occur in these mice if diazepam was infused in the visual cortex during the critical period. High [^{11}C]flumazenil volume of distribution in early childhood as demonstrated by PET scanning (Chugani et al., 2001) may be related to a specific role of benzodiazepine-sensitive $GABA_A$ receptors in critical periods of synaptic plasticity during human brain development. Pairing of visual sensory experience with the lengthening of the critical period may be a powerful way to provide a therapeutic intervention in this system. Similarly, serotonergic drugs have been shown to alter the thalamocortical connectivity of the somatosensory cortex as described above. Pairing of various sensory interventions with pharmacological treatment impacting serotonergic tone may improve the efficacy of this type of intervention.

Conclusion

A new era of pharmacological interventions in autism is possible due to increasing understanding of molecular events regulating critical periods of brain development.

In addition, the ability to ascertain the genetic variations in individuals with autism to determine which developmental process may be impacted might guide rational strategies for intervention. Use of pharmacological strategies, paired with behavioral interventions, holds promise for impacting brain function in children with autism.

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Glutamic Acid Decarboxylase (GAD) as a Biomarker of GABAergic Activity in Autism: Impact on Cerebellar Circuitry and Function

Gene J. Blatt, Jean-Jacques Soghomonian, and Jane Yip

Experimental evidence implicates the inhibitory neurotransmitter γ-amino butyric acid (GABA) in the pathophysiology of autism. For example, a reduced density of $GABA_A$ and benzodiazepine receptors in the hippocampus (Blatt et al., 2001; Guptill et al., 2007), reduced $GABA_A$ and $GABA_B$ receptor protein subunits in cerebellum, prefrontal Brodmann area 9 and parietal Brodmann area 40 (Fatemi et al., 2009a, b), and a reduced expression of the GABA synthesizing enzymes GAD65 and GAD67 in the parietal and cerebellar cortex (Fatemi et al., 2002) have been documented in autistic brains. More detailed anatomical studies have shown that GAD67 and/or GAD65 gene expression is decreased in Purkinje cells and increased in Basket cells in the cerebellum in autism, whereas GAD65 is decreased in the dentate nucleus of brains from autistic patients (Yip et al., 2007, 2008, 2009). A dysfunction of GABA in autism is further supported by the effectiveness of $GABA_A$ receptor agonists in treating seizure and anxiety disorders in patients (Askalan et al., 2003; Acosta, 2004). The possibility that these GABAergic alterations are involved in the development of autism rather than being a consequence of the disorder is supported by genetic studies that have found an interstitial duplication of chromosome 15q11.2-q13, a region containing three $GABA_A$ receptor subunits (α5, β3, γ3) (Schroer et al., 1998; Shao et al., 2003; Cook et al., 1997; Mohandas et al., 1999; Boyar et al., 2001; Nurmi et al., 2001). Recently, *GABRB3* has been implicated as a risk allele in autistic families on the same chromosome (Buxbaum et al., 2002; Curran et al., 2005). Collins et al., (2006) recently also implicated *GABRA4* and *GABRB1* as genes that contribute to autism susceptibility. It is now apparent that the genetic architecture in autism is highly variable consisting of both multiple rare and common variation (Ma et al., 2009). In this chapter, we will present and discuss the experimental evidence supporting an alteration of GABAergic activity in autism with a focus on cerebellar circuitry. Specifically, alterations in GABAergic biomarkers *within* neuronal subtypes are demonstrated in fresh-frozen postmortem cerebellar samples via in situ hybridization techniques.

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Glutamic Acid Decarboxylase Activity Is Involved in the Modulation of GABA Levels

GABA is synthesized from glutamate through the activity of the rate-limiting enzyme glutamic acid decarboxylase (GAD). In the mammalian brain, at least two highly conserved isoforms of GAD are present and expressed by a majority of GABAergic neurons (Mercugliano et al., 1992). These two isoforms are known as GAD65 and GAD67 because their relative molecular weights are 65 and 67 kilodaltons (kDa), respectively. The two GAD isoforms presumably originated in vertebrates following gene duplication approximately 450 million years ago before the emergence of sharks and rays (Lariviere et al., 2002). There is a third form of GAD, GAD3, that has been evidenced in the fish species *Coryphaenoides armatus*, the deep sea armed grenadier, and the gold fish (Lariviere et al., 2002). In addition, alternate spliced variant products of the GAD67 gene have been evidenced but are not expressed in the adult brain (Chessler and Lernmark, 2000).

Although the two GAD isoforms are co-expressed in a vast majority of GABAergic neurons, they exhibit a different affinity and interaction with the co-factor pyridoxal-5′-phosphate, they are distributed in different intracellular compartments, their transcriptional regulation involves different mechanisms and they contain different phosphorylation sites, which modulate their activity to a different extent. In particular, within neurons, GAD67 appears to be mainly holoenzyme (bound to the co-factor) ,whereas GAD65 is largely apoenzyme (requires a co-factor but does not have one bound) (Battaglioli et al., 2003). Thus, the apo-/holoenzyme cycle of GAD inactivation and reactivation is more important in regulating the activity of GAD65 than GAD67 (Pedersen et al., 2001; Battaglioli et al., 2003). The contribution of each GAD isoform to the relative amount of GABA in the brain is illustrated by studies in GAD67- or GAD65-deficient mice. Deletion of the GAD65 gene does not significantly alter brain GABA levels (Asada et al., 1997a). In contrast, GAD67 gene deletion results in mice born with much lower GABA content and these mice are not viable postnatally because of their inability to feed due to a cleft palate (Asada et al., 1997b). These gene deletion studies are consistent with the idea that GAD67 is predominantly in an active form in vivo and contributes to the synthesis of a larger pool of GABA when compared to GAD65. Gene deletion studies also provide compelling evidence that the two GAD isoforms are involved in the biosynthesis of a pool of GABA used for neurotransmission. GAD65-deficient mice exhibit a number of behavioral and cellular abnormalities including enhanced susceptibility to seizure (Kash et al., 1997), increased anxiety-like responses in open field and elevated zero maze assays (Kash et al., 1999), altered conditioned fear behavior (Stork et al., 2003), loss of ocular dominance plasticity (Faglioni and Hensch, 2000, Iwai et al., 2003), deficits in prepulse inhibition (Heldt et al., 2004), and altered taste function (Shimura et al., 2004). In these mice, the size and the frequency of GABA-mediated spontaneous inhibitory postsynaptic currents (IPSCs) in the retina or hippocampus are not altered but the release of GABA during sustained synaptic activation is substantially reduced (Tian et al., 1999). This

suggests that although GAD65 is required for normal GABA function, the GAD67 isoform also plays a central role in the biosynthesis of a pool of GABA used for GABAergic neurotransmission. Earlier studies have shown that the GAD67 mRNA is more abundant in striatal and cortical GABAergic neurons known to have a tonic firing activity when compared to other neurons such as striatal projection neurons, which are known to fire action potentials phasically (Feldblum et al., 1993). This observation led authors to propose that GAD67 could be specialized in the production of constant GABA levels for release by tonically firing GABAergic neurons. In contrast, other studies have shown that GAD65 directly interacts with synaptic vesicles (Hsu et al., 1999; Tian et al., 1999; Hsu et al., 2000) suggesting that this isoform may be preferentially involved in the biosynthesis of a pool of GABA used for vesicular release. However, neurons in mice with a deletion of the GAD65 gene are still able to accumulate and release GABA through a vesicular mechanism (Wu et al., 2007), which indicates some overlap in the function of GAD65 and GAD67.

GABA as a Developmental Factor

In addition to its role as an inhibitory neurotransmitter, GABA is also involved in neural development and circuit formation (Ben-Ari et al., 1997). GABA exerts trophic effects on neuronal migration and neurite outgrowth during the embryonic and perinatal stages, an action explained in part by its depolarizing effect on immature neurons (Ben-Ari et al., 1989). More recent evidence also shows that GABA is specifically involved in the development of perisomatic inhibitory synapses in the somatosensory cortex (Chattopadhyaya et al., 2004; 2007). As documented in in vitro preparations such as organotypic cultures (Ji and Obata, 1999) as well as in vivo models, the neurodevelopmental effects of GABA involve a specific role of GAD67. In particular, conditional knockdown of GAD67 in basket interneurons in adolescent visual cortex results in deficits in axon branching, perisomatic synapse formation around pyramidal neurons and complexity of innervation fields, and germline knockdown of GAD67, but not GAD65, results in similar deficits (Chattapodyaya et al., 2007). The role of GAD67 in circuit development is subtle since hippocampal and cerebellar organotypic cultures taken from GAD67-deficient mice develop an extensive GABA-containing fiber network (Ji and Obata, 1999), suggesting that the GAD65 isoform can compensate for the lack of GAD67. A more recent study in the mouse cerebellum, however, indicates that selective GAD67 deletion using the Cre-loxP strategy impairs motor coordination as assessed on the rotarod and reduces the frequency and amplitude of mIPSCs in Purkinje cells (Obata et al., 2008). In the human cerebellum, both GAD65 and GAD67 mRNA are strongly expressed during development but the two isoforms differ in their timing of expression. GAD65 and GAD67 mRNA can be detected at gestational week 12 but GAD67 mRNA levels remain high for the rest of the gestational period whereas GAD65 mRNA levels decrease rapidly from week 12 to 19 (Chan et al., 1997). In spite of the apparent low activity of GAD65 in subsequent fetal life, GAD65 is

important in maintaining GABA levels and could offset a deficiency in GAD67. A specific role of GAD67 in neuronal plasticity and neurodevelopmental processes is further supported by evidence that expression of this isoform is strongly experience driven. The activity of GAD67 coincides with its long-term upregulation of protein and mRNA levels during intense neuronal activity such as stress- and drug-induced stimulation in various brain regions (Bowers et al., 1998), drug-induced seizures (Freichel et al., 2006), and during modulation of neuronal activity (e.g., Benson et al., 1989; Soghomonian et al., 1992; Benevento et al., 1995; Liang and Jones, 1996; Gierdalski et al., 2001; Kobori and Dash, 2006).

Regional Distribution of GAD in Cerebellar Interneurons

There are three major classes of inhibitory interneurons in the cerebellar cortex (Chan-Palay, 1977; also see Fig. 1). Basket and stellate cells are localized in the molecular layer and Golgi neurons in the granule cell layer. The inhibitory nature of stellate and basket cells was clarified in the 1960s (Eccles et al., 1967; Andersen et al., 1964) and when antibodies became available for GAD, it was found that each class of inhibitory interneuron contained GAD (McLaughlin et al., 1974; Saito et al., 1974; Ribak et al., 1978; Oertel et al., 1981). More recent studies with antibodies recognizing GABA itself provided direct evidence that GABA is present in basket, stellate, and Golgi cells (Ottersen and Storm-Mathisen, 1984; Seguela et al., 1985; Somogyi et al., 1985, Gabbott et al., 1986). These neurons also have the ability to accumulate GABA via a membrane transporter, as shown originally in autoradiographic studies following ^{3}H-GABA uptake (Hokfelt and Ljungdahl, 1970; Schon and Iversen, 1972), and more recently through the immunohistochemical demonstration of the carrier protein itself (Radian et al., 1990). Cerebellar glial cells are similarly endowed with GABA uptake mechanisms (Radian et al., 1990) and may assist the neurons in terminating transmitter action. The simple concept that all cerebellar interneurons are purely GABAergic was challenged by the finding of Wilkin et al (1981) of high-affinity glycine uptake in a subpopulation of Golgi cell terminals. In recent years it has become clear that a substantial proportion of Golgi cells (70% or more) are enriched in GABA as well as glycine, as assessed by antibodies that selectively recognize these two amino acids (Ottersen et al., 1988). Colocalization occurs both in the cell bodies and axon terminals.

Cerebellar Circuitry, Function, and the Region of Interest: Crus II Region in the Posterior Lobe in the Lateral Hemisphere

The normal cerebellar circuitry is illustrated and described in Fig. 1. In the present studies labeling of GAD mRNA was performed via in situ hybridization and quantified with the software program NIH Image J. The studies reported here focused on specific GABAergic neuronal types in the cerebellum: Purkinje cells (PCs) in the

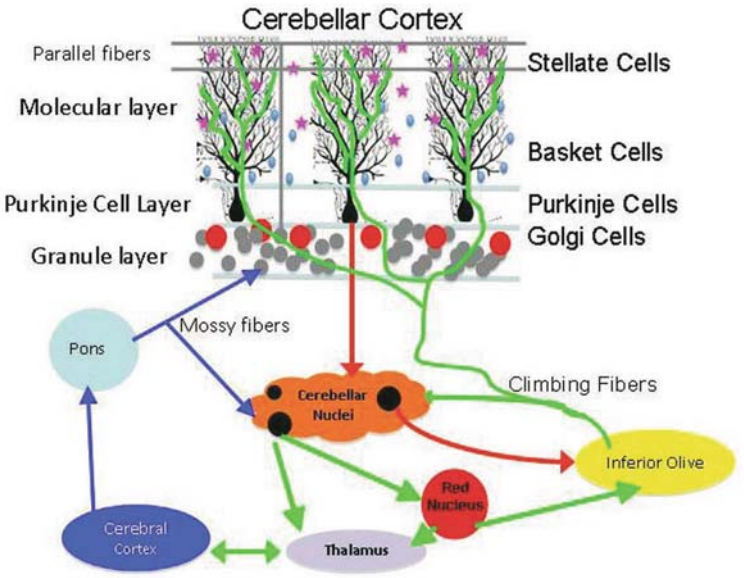

Fig. 1 Normal cerebellar circuitry. Excitatory afferents arise from the inferior olive (climbing fibers) and other precerebellar nuclei (e.g., pons) as mossy fibers. The pons receives widespread input from the cerebral cortex. Olivocerebellar climbing fibers (glutamate/aspartate) project directly to GABAergic Purkinje cells and send collaterals to the cerebellar nuclei. Mossy fibers (glutamate) also send collaterals to the cerebellar nuclei and project to Golgi cells and granule cells which in turn send parallel fibers (glutamate) to Purkinje cell dendrites in the molecular layer. GABAergic stellate and basket cells in the molecular layer also receive parallel fiber input and innervate Purkinje cells. Golgi cells in the granule layer project their axons to granule cells. Cerebellar nuclei send their output via the thalamus and red nucleus and then back to the cerebral cortex. GABAergic subpopulations in the cerebellar nuclei are thought to send feedback projections to the inferior olive and/or innervate other cerebellar nuclear cells (e.g., dentate nuclei).

PC layer, stellate and basket cells in the molecular layer, Golgi cells in the granular layer, and subpopulations of presumed inhibitory neurons in the dentate nuclei. The studies were performed in postmortem tissue samples taken below the horizontal fissure in the Crus II region of the lateral hemisphere (see Schmahmann et al., 2000). This area was chosen for two main reasons: (1) the lateral hemisphere is the region of the greatest decreases in Purkinje cells in studied subjects with autism (see Whitney et al., 2008 for review) and (2) it is a main recipient region for frontopontine projections to the cerebellar cortex which is robust in humans compared to non-human primates (see Ramnani, 2006). The lateral hemisphere is most recently evolved in humans and apes and receives input almost exclusively from cerebral cortex via pons (i.e., "cerebrocerebellum"). Purkinje cells project to the dentate nucleus then primarily to the ventrolateral nucleus of the thalamus and on to premotor, motor, and prefrontal cortices. Functionally it is involved in planning and mental

rehearsal of complex motor actions, movement errors (e.g., Eccles et al., 1967), and via the olivocerebellar system, motor learning (Llinas and Welsh, 1993; Ito, 1993a), and thought/cognition (e.g., Ito, 1993b, c; Schmahmann, 2001a, b). Specifically, the cerebellum combines sensory information from spinal cord and sensory systems with input about initiation (planning) of movements from cerebral cortex and compares and integrates information and projects to descending motor systems to coordinate smooth appropriate movements. Cerebellar lesions can result in movements falling short of intended targets (dysmetria), jerky, or erratic movements and ataxia which is not always present. Its widespread input from cerebral cortical areas via the pontine nuclei allows integration and coordination of higher order behaviors including working memory and executive function tasks. Lesions in the dentate nucleus in monkeys result in delays in a visual recognition span test (Schmahmann et al. 2004). Other cognitive deficits with cerebellar lesions include visuospatial deficits, limitations in cognitive ability, linguistic deficits, emotional blunting and psychological distress, i.e., "dysmetria of thought and emotion" – the "Cerebellar Cognitive Affective Syndrome" (Schmahmann, 2001a, b).

In the next section we report findings in GAD mRNA levels in specific neuronal types within the cerebellar circuitry in the Crus II region. Much has been recently published (Yip et al, 2007, 2008, 2009) with new results reported here for Golgi cells labeled with a probe for GAD65 mRNA.

GAD Abnormalities in the Cerebellum in Autism

GAD67 mRNA Levels in Purkinje Cells in the Crus II Region

In autism, a decrease in GAD67 protein levels as measured in whole cerebellar homogenates was initially reported (Fatemi et al., 2002), suggesting a decrease in GABAergic neurotransmission in the cerebellum. More recently, anatomical studies with in situ hybridization histochemistry have shown that GAD67 mRNA levels are also markedly decreased in Purkinje cells of autistic brains (Yip et al., 2007) (Fig. 2; $p<0.0001$, two tailed *t*-test).

GAD67 mRNA Levels in Cerebellar Basket and Stellate Cells in the Crus II Region

In contrast to Purkinje cells, cerebellar basket cells (BC) had a higher mean level of GAD67 mRNA (Yip et al., 2008; $p<0.0001$, independent *t*-test) in the autism group compared to the control group. GAD67 mRNA levels in stellate cells were not significantly different between controls and autistic brains but did show a trend for significance for an increased level.

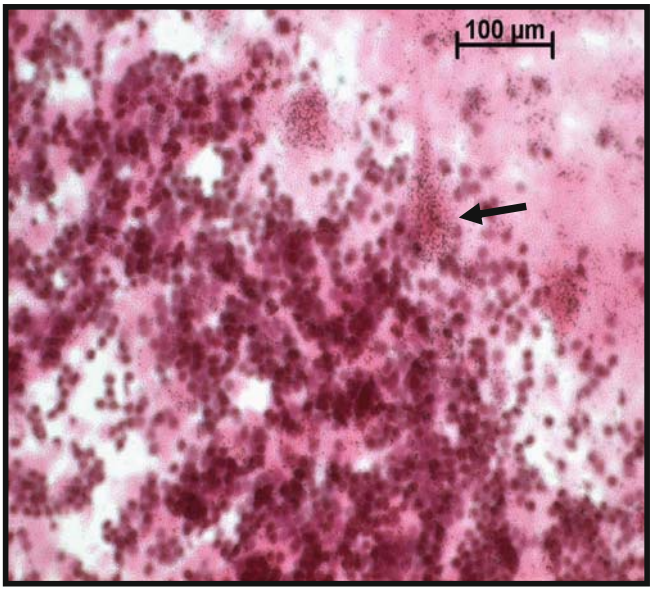

Fig. 2 Glutamic acid decarboxylase (GAD) 67 mRNA-labeling in cerebellar Purkinje cells in the Crus II region of the lateral cerebellar hemisphere from an individual with autism. Note the accumulation of silver grains within the soma and apical dendrite (*arrow*) labeled via in situ hybridization. Scale bar = 100 µm

GAD67 mRNA Levels in Golgi Cells in the Crus II Region

GABAergic Golgi type II cells play important roles in cerebellar function directly influencing excitatory parallel fibers and granule cells and the final inhibitory/excitatory balance in Purkinje cells. In situ hybridization studies showed that the level of GAD67 mRNA was not significantly different between autistic and control subjects (Fig. 3; 2.2 ± 0.23 pixels/μm^2 autism; 1.97 ± 0.12 control; pixels/μm^2; independent *t*-test, $p = 0.10$).

GAD65 mRNA Levels in the Dentate Nucleus

In a recent in situ hybridization study, a 51% reduction in GAD65 mRNA levels was observed in a subpopulation of large neurons in dentate nucleus in five adult autism cases compared to six age- and PMI-matched controls (Yip et al., 2009). In contrast, in the same study, non-significant GAD65 mRNA levels were measured in a subpopulation of smaller dentate neurons in the same postmortem cases.

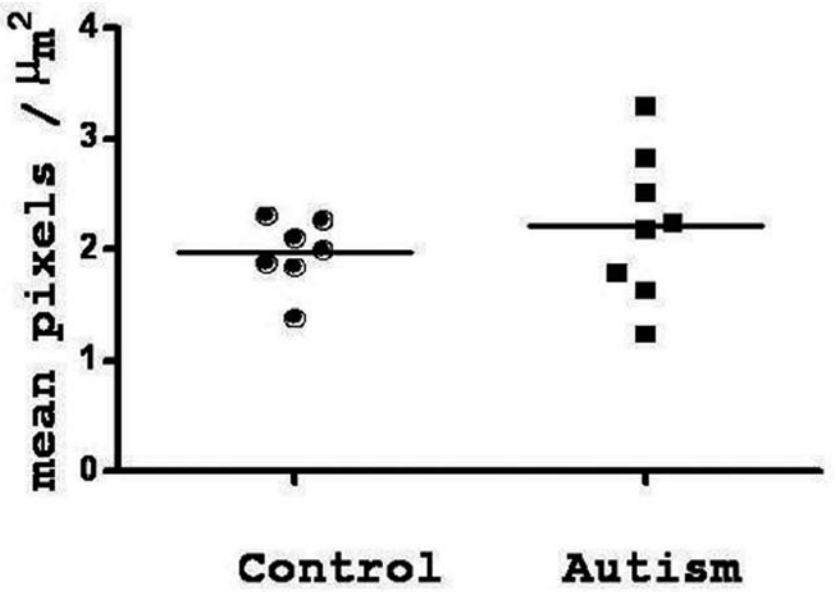

Fig. 3 Scatter plot showing the mean levels of GAD67 mRNA-labeling in Golgi cells in seven control cases (*circles*) and eight subjects with autism (*squares*) sampled from 60 cells per subject (1.97 ± 1.17 pixels/μm^2 control; 2.21 ± 2.36 pixels/μm^2 autism). Statistical analysis did not show a significant difference in GAD67 mRNA levels in the autistic group compared to age-, PMI-, and pH-matched controls (student *t*-test and Levene's test for equality of variances: $F = 2.981$; $p = 0.401$ control, and $p = 0.384$ autism)

Altered GAD in the Lateral Cerebellar Hemisphere May Impact the Normal Functioning of Cerebellar Circuitry in Autism

The normal functioning of the cerebellar cortex is dependent on its excitatory inputs from olivocerebellar climbing fibers (CFs) that have glutamate/aspartate as its transmitters as well as the glutamatergic mossy fiber (MF)/parallel fiber (PF) inputs to granule cells and Purkinje cells, respectively. It is not known whether the precerebellar inputs from the pontine nuclei and other spinal and brainstem nuclei are altered in autism. Detailed studies of the pons is especially warranted due to its abundant inputs from the cerebral cortex via the fronto and parietopontine projections that are in turn relayed to granule cells via collaterals to the cerebellar nuclei. It is also important to study the pontine nuclei because of its relay of frontopontine projections to the lateral hemisphere in the posterior lobe to the Crus I and II regions with emphasis on the latter recipient area (Ramnani, 2006). Regarding CFs from the inferior olivary complex, this olivocerebellar projection travels through the contralateral inferior cerebellar peduncle terminating on Purkinje cell (PC) soma and primary, secondary, and tertiary dendrites in a 1:1 ratio, i.e., each PC receives only

one CF. This is in contrast to each PC being innervated by up to 200,000 parallel fibers in the molecular layer as demonstrated in rodents (Chan-Palay, 1977). It is thought that the CF input is important for the timing of PC firing whereas the MF/PF inputs likely play a role in the magnitude of the response.

One of the key findings from our GAD studies is that the net result of the intrinsic GAD changes in the cerebellum in the autism group is that there is a significant decrease in GAD65 mRNA levels in the dentate nucleus. This is important because there are different subpopulations of neurons that compose the dentate nucleus (Chan-Palay, 1977; Oertel et al., 1981; Sultan et al., 2003). The smallest cells (about 10 μm) are thought to be intrinsic interneurons that innervate the larger dentate neurons whereas the larger dentate neurons (about 20 μm) are composed of at least two subgroups, an inhibitory and an excitatory subgroup (Chan-Palay et al., 1977). The small neurons and some larger neurons contain GAD but the majority of large neurons (about 20–30 μm) do not and are presumably glutamatergic. Some inhibitory interneurons also co-localize with glycine in rodents. The GAD65 mRNA deficit in levels were found in the larger inhibitory population that in animal studies are thought to project back to the inferior olivary complex (Tolbert et al., 1978; Oertel et al., 1981; Nelson and Mugnani, 1989; Saint-Cyr and Courville, 1981; De Zeeuw et al., 1988, 1996, 1998). Less feedback inhibition to the inferior olive, and specifically to the principal olive that is targeted by the dentate, could have major implications to the normal functioning of the structure. Specifically, inferior olivary neurons are electrotonically coupled and fire in synchronous patterns activating groups of PCs across lobules. Asynchronous firing of olivary neurons can result in alterations of the firing pattern of PCs that in turn can impact neurons in the dentate. An added concern is the neuropathologic description of the inferior olive in autism cases (see Welsh, 2002). Both Bailey et al. (1998) and Bauman and Kemper (1985; 1994) described developmental abnormalities in the inferior olive in some autism cases including ectopic neurons and migrational abnormalities. Two groups have additionally described ectopic principal olive neurons as abnormal alignment along the edges of the olivary ribbon (Bauman and Kemper, 1985; Thevarkunnel et al., 2005). This could result in affected olive cell dendrites to be mis-aligned with possible effects on the synchrony of firing. Further, we have recently found that some CFs are themselves abnormal in autism cases with an abnormal accumulation of class II intermediate neurofilaments in their projection axons via immunolabeling with peripherin (Blatt et al., 2007). It is unknown whether this morphological abnormality might impact the release of glutamate/aspartate onto PC dendrites. If so, the excitation:inhibition balance may be further disturbed.

Overall, these studies demonstrate the cerebellum and olivocerebellar system are impacted at multiple points within the circuitry (see Fig. 4) suggesting that these structures are extremely vulnerable throughout the projection/processing regions of the structures. Unless there are multiple points of compensatory changes all along the paths, one can conclude that the normal functioning of the circuitry in the Crus II region is strongly impacted with likely consequences further downstream. Whether thalamo-cortical circuits to motor and prefrontal areas suffer the consequences of these changes is unknown. In the motor system these changes could

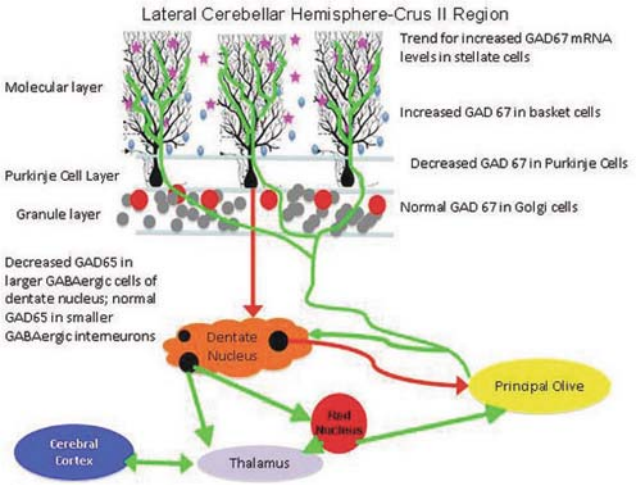

Fig. 4 Cerebellar circuitry diagram illustrating quantitative changes in GAD65 and 67 mRNA in specific neuronal types in the lateral cerebellar hemisphere, Crus II region in autism. Decreased GAD67 mRNA was measured in Purkinje cells and decreased GAD65 mRNA was found in a larger subpopulation (18–20 μm average size) of GABAergic dentate cells. In contrast, increased GAD67 mRNA was measured in molecular layer interneurons and normal GAD mRNA levels were found in Golgi cells (GAD67) and in a smaller subpopulation (10 μm average size) of dentate neurons (GAD65)

impact the modulation and error-correction from commands from primary motor cortex. Similarly, in the cognitive domain, there could be alterations in the modulation of frontal lobe output. Behaviorally, stereotyped-repetitive behaviors to those of executive function and working memory may be impacted. This hypothesis is based on a growing amount of evidence that a structure that contains over half of all the neurons in the brain, the cerebellum, is impacted at multiple points along its extrinsic and intrinsic circuitry.

Additional Functional Considerations

The exact functional significance, origin, or timing of alterations in GAD gene expression in the cerebellum of individuals with autism is still unclear. In particular, it is unclear if these alterations are a consequence or a cause of the disease process. A number of degenerative diseases of the CNS, such as stiff-person syndrome (SPS), progressive cerebellar ataxia, and Rasmussen encephalitis, have been characterized by the presence of autoantibodies and recent findings suggest that Batten disease may be associated with autoantibodies against GAD (Vianello et al., 2002; Pearce et al., 2004). Anti-GAD autoantibodies could result in excess excitatory neurotransmission, leading to seizures and other symptoms observed in patients with Batten disease (Vianello et al., 2002; Pearce et al., 2004). The hypothesis that autism may

also involve the production of autoantibodies against GAD has been recently been proposed (Rout and Dhossche, 2008). Studies in a number of disorders including diabetes type I indicate that the GAD65 isoform is much more antigenic than the GAD67 isoform (Fenalti and Rowley, 2008). Based on these findings, it is tempting to speculate that the decrease in GAD65 expression in the cerebellar nuclei (i.e., dentate) in autism may be linked to the presence of autoantibodies. Other studies have shown that a decrease in GAD activity or GAD65 immunolabeling in nerve terminals can be induced prenatally by chronic maternal ethanol consumption (Bailey et al., 2004) or by the exposure to toxic environmental agents such as cisplatin, a cytotoxic agent to the developing cerebellar cortex (Scherini and Bernocchi, 1994; Pisu et al., 2003). Thus, prenatal events other than autoantibodies could also lead to alterations in GAD expression in autism. Once present, however, and as discussed in previous paragraphs, such alterations in GAD expression may have a negative impact not only on the inhibitory mechanisms in the brain but also on the neurogenesis and/or development of normal GABAergic neuronal circuits. The data presented and discussed in this chapter raise a number of important questions about the origin and the functional consequences of alterations in GAD gene expression in autism. Clearly, further investigations are needed to determine if these alterations in GAD gene expression are linked to the disease process or are a consequence of it.

Concluding Notes and Ongoing Studies

The evidence is mounting that the GABAergic system may play a major role in the autistic disorder. Multiple biomarkers have emerged in the cerebellum that demonstrate consistent and significant alterations in the key synthesizing enzymes for GABA, GAD65, and GAD67 that may directly affect GABA innervation to target neurons within the circuitry. Effects on the cerebellar nuclei (i.e., dentate) may have profound effects on projections to prefrontal cortical areas with potential disruptions in the modulation and/or error correcting of motor signals and cognitive behaviors.

Beyond the cerebellum, GABA abnormalities have recently been demonstrated in the hippocampal formation in interneuron packing density (Lawrence et al., 2009, in press) and $GABA_A$ receptors and benzodiazepine binding sites (Blatt et al., 2001; Guptill et al., 2007), and decreases in the same receptors/binding sites were found in the anterior cingulate cortex (Oblak et al. 2009, in press). Studies are now underway in our laboratory to determine whether changes in GABA interneurons and receptors are more widespread beyond limbic and cerebellar regions and into neocortical areas in the autism brains. We are also investigating changes in other types of GABA receptors (e.g., $GABA_B$) and key $GABA_A$ receptor subunits. Finally, additional studies are underway investigating the glutamatergic system in the cerebellum and beyond to determine how the excitatory:inhibitory synaptic balance may be altered in the brains of individuals with autism. It is our hope that the ultimate result of these studies will yield valuable information regarding specific biomarkers for the

development of targeted pharmacotherapies or gene therapies to improve the lives of affected individuals.

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Epigenetic Dysregulation of 15q11-13 $GABA_A$ Receptor Genes in Autism

Amber Hogart and Janine M. LaSalle

$GABA_A$ Receptor Function and Genome Organization

Role of GABA in Brain Function

GABA, γ aminobutyric acid, is the major inhibitory neurotransmitter in the mammalian brain. GABA is found in nearly all organisms (Jelitai and Madarasz, 2005) and has evolved diverse functions in important physiologic processes such as stress response in plants (Shelp et al., 1999) and motor behaviors in insects (Leal et al., 2004). In mammals, GABA is synthesized from glutamate by two distinct glutamic acid decarboxylases, GAD65 and GAD67 (Erlander et al., 1991). Although GABA functions to hyperpolarize the membrane in the adult mammalian brain, in early development GABA signaling results in membrane depolarization due to high intracellular concentration of chloride (Cl–) ions. Early membrane depolarization, prior to synapse formation, leads to influx of calcium (Ca++), with downstream effects inducing neuronal development (Ben-Ari, 2002; Fiszman and Schousboe, 2004).

GABA neurotransmission is mediated by two classes of receptors, fast ionotrophic $GABA_A$ receptors and slow metabotrophic $GABA_B$ receptors. The classification of GABA receptors was originally based on the pharmacology of the intact receptors, with "classical" $GABA_A$ receptors responding to the GABA analogue bicuculline and bicuculline-insensitive $GABA_B$ receptors responding to baclofen (Bowery, 1989). Later discoveries of a class of GABA receptors insensitive to both bicuculline and baclofen led to the classification of an additional group of GABA receptors unofficially termed $GABA_C$ receptors (Bormann, 2000). $GABA_A$ receptors are generally heteropentamers of three main classes of subunit genes, α, β, and γ, that when assembled form an ion channel that enables a fast inhibitory response. Although structurally similar to other $GABA_A$ receptors, $GABA_C$ receptors are formed exclusively by the rho subunit (ρ1–3) and are preferentially found in the retina (Bormann, 2000). Cloning of GABA receptor mRNA has revealed

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that $GABA_B$ receptors are heterodimers of two G-protein-coupled receptor genes $GABA_B$ R1 and $GABA_B$ R2 (Blein et al., 2000).

Genomic Clustering of the $GABA_A$ Receptor Genes in the Human Genome

Thorough examination of the human genome sequence has revealed that 19 different $GABA_A$ receptor subunit genes are present in humans, making the $GABA_A$ receptors the largest set of ion channel receptors in mammals (Simon et al., 2004). $GABA_A$ receptors are organized into classes based on sequence similarity, with subunits of the same class sharing 60–80% sequence identity while subunits of different classes typically share only 30–40% sequence similarity (Darlison et al., 2005). There are eight classes of $GABA_A$ subunits, α (1–6), β (1–3), γ (1–3), δ, ϵ, θ, π, and ρ (1–3). Most subunits are present in genomic clusters containing at least one α, β, and γ (Bailey et al., 1999; Russek, 1999). Figure 1 illustrates the genomic locations of the $GABA_A$ receptor clusters on chromosomes 4, 5, 15, and X. Interestingly, the cluster of $GABA_A$ receptor genes on Xq28 does not contain β and γ subunits, but instead contains the θ and ϵ subunits which are most similar (45–50% amino acid identity) to the chicken β4 and γ4 genes (Sinkkonen et al., 2000; Simon et al., 2004).

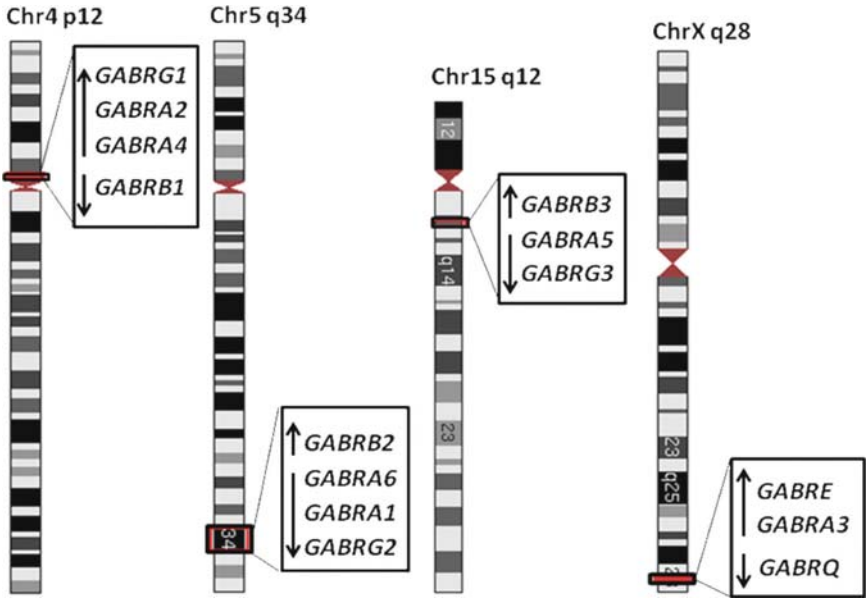

Fig. 1 Genomic clustering of $GABA_A$ receptor subunit genes (recreated from UCSC genome browser)

Conserved clustering of $GABA_A$ receptor genes has led to the hypothesis that the physical proximity of receptor subunit genes facilitates temporal and/or spatial coordinate expression, however, limited evidence exists to support this claim (Steiger and Russek, 2004). Studies of $GABA_A$ receptor mRNA distribution in adult rat brain have revealed that the α1-β2-γ2 gene cluster represent the most abundant receptor subunits, however, the α6 subunit, also present in the cluster, is one of the least prevalent subunits in the brain (Laurie et al., 1992; Wisden et al., 1992; Pirker et al., 2000). Furthermore, mRNA expression analysis of *GABRB3*, *GABRA5*, and *GABRG3* genes colocalized to chromosome 15 in humans, demonstrated that the expression levels of these genes are not comparable in frontal cortex. While *GABRB3* mRNA was abundant in post-mortem tissue samples from adult cortex samples, *GABRA5* and *GABRG3* were barely detectable (Hogart et al., 2007).

Phenotypic Consequences of Disruption of $Gaba_a$ Receptors

Imbalances in the GABAergic system have been implicated in a multitude of neurophysiologic processes including anxiety, memory, insomnia, ataxia, and seizures (Mohler, 2006). $GABA_A$ receptor subunit genes have highly heterogenous expression and distribution in the brain (Pirker et al., 2000), therefore systematic analysis of subunits by mouse knockout models has been useful for assessing the role of individual subunit genes. Although not all subunits have been knocked out in mice, knockouts of the most highly expressed subunits including α1, β2, β3, and γ2 have been created. The most severe phenotypes were observed in the β3, and γ2 knockout mice, where the vast majority of mice were neonatal lethal, while α1 and β2 knockout mice had surprisingly few detrimental phenotypes (Rudolph and Mohler, 2004). Examination of the intact $GABA_A$ receptors in knockout mice has shown that α1, β2, and β3 knockouts have only 50% of normal numbers of $GABA_A$ receptors, while γ2 knockout mice have normal numbers of receptors, revealing that γ subunits are not required for receptor assembly (Rudolph and Mohler, 2004). Furthermore, one of the most important revelations from knockout mice studies is the degree to which dysregulation of a single subunit, such as α1, can result in compensatory changes in expression of other subunit genes. The high degree of coordinate control between different subunits likely contributes to the complexity of phenotypes in different knockout models (Rudolph and Mohler, 2004).

Chromosome 15q11-13 and Neurodevelopmental Disorders

Human chromosome 15q11-13 is subject to complex genomic rearrangements resulting in various neurological outcomes depending on the type of rearrangement and the parental origin of the chromosome. Interstitial deletions, duplications, and supernumerary marker chromosomes (also called isodicentric chromosome 15) are formed due to unequal meiotic recombination of low copy repeats (LCRs)

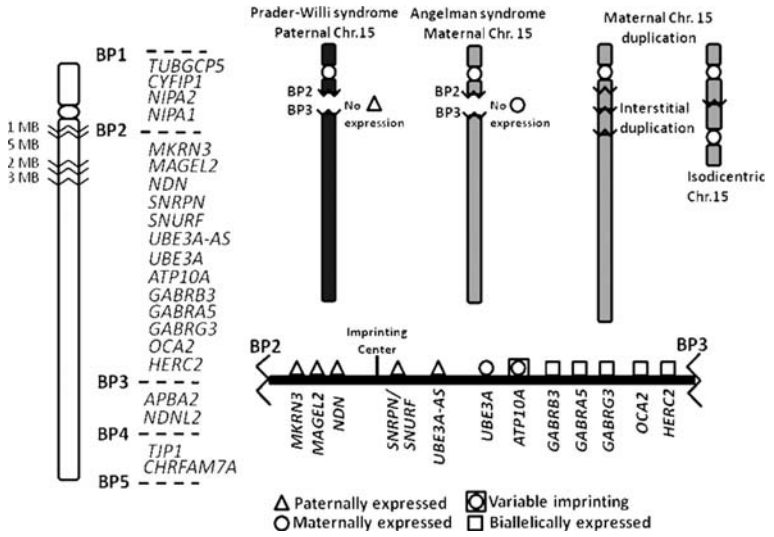

Fig. 2 Common breakpoints in chromosome 15q11-13 and genes included within the Prader–Willi syndrome Angelman syndrome critical region (PWACR) from BP2-BP3

at five common breakpoints throughout the approximate 11 MB region (Fig. 2) (Wang et al., 2004). The resulting phenotype is dependent on the parental origin of the rearrangement because a cluster of genes within 15q11-13 are subject to parent-of-origin specific gene expression, a process termed genomic imprinting. Imprinting was discovered in the 1980s after mice with entirely maternal or paternal genomes failed to develop (McGrath and Solter, 1984; Surani et al., 1984). Parental imprints are established in germ cells by epigenetic modifications such as DNA methylation. Parent-specific gene expression of the 15q11-13 imprinted genes is maintained by regulatory sequences called imprinting control regions for maternal and paternal chromosomes. This bipartite imprinting center influences the expression of genes both upstream and downstream over a long range (Reik and Walter, 2001).

Chromosome 15q11-13 contains paternally expressed genes (maternally imprinted), maternally expressed genes (paternally imprinted), as well as biallelically expressed genes (Fig. 2). Absence of the paternally expressed genes leads to Prader–Willi syndrome while absence of the maternally expressed genes leads to Angelman syndrome. The paternally expressed genes are methylated on the maternal chromosome and are consistently silenced in all tissues. The maternally expressed imprinted gene, *UBE3A*, differs from the paternally expressed genes because paternal silencing appears to be independent of DNA methylation and is restricted to neurons (Albrecht et al., 1997; Rougeulle et al., 1997; Yamasaki et al., 2003). Although the 15q11-13 $GABA_A$ receptor (GABR) genes are not imprinted in mice (Nicholls et al., 1993; Buettner et al., 2004), several studies have suggested that these genes may have paternal expression bias in humans (Meguro et al.,

1997; Bittel et al., 2003; Bittel et al., 2005). Examination of the imprinting status of the 15q11-13 GABR genes in human brain, however, demonstrated that these genes are normally equally expressed from both parental chromosomes (Hogart et al., 2007).

Prader–Willi and Angelman Syndromes

Prader–Willi syndrome (PWS) is a neurological disorder caused by paternal deficiency of 15q11-13. The most common genetic defects in PWS are a deletion of the paternal chromosome (~70%) and uniparental disomy (UPD) of the maternal chromosome (~25%), while a rare cause involves defects of the imprinting center (<5%) (Nicholls and Knepper, 2001). PWS is characterized by hypotonia at birth followed by obesity in childhood due to hyperphagia, mild mental retardation, and behavior problems (Bittel and Butler, 2005). Angelman syndrome (AS), a more severe neurodevelopmental disorder, is most commonly caused by a deletion of maternal chromosome 15q11-13 (~70%), and more rarely is caused by paternal UPD (~5%) and imprinting defects (~5%) (Nicholls and Knepper, 2001). Mutations in the maternally expressed imprinted gene *UBE3A* have also been shown to cause AS and account for ~10% of cases (Kishino et al., 1997; Matsuura et al., 1997). Classic features of AS include severe developmental delay, absence of speech, mental retardation, ataxia, and unique behaviors including happy demeanor with frequent smiling and laughing (Clayton-Smith and Laan, 2003). In both AS and PWS, patients with 15q11-13 deletions have more severe cognitive impairment and a much higher incidence of seizures than UPD cases suggesting that nonimprinted genes, including the GABR subunit genes, contribute significantly to the clinical manifestations of these neurodevelopmental disorders (Varela et al., 2004, 2005; Torrado et al., 2007).

Rett Syndrome

Rett syndrome (RTT) is a severe neurodevelopmental disorder that is classified as one of the five pervasive developmental disorders. RTT is characterized by normal early development followed by regression and loss of developmental milestones around 6–18 months of age, absence of speech, severe mental retardation, loss of purposeful hand movements, and ataxia (Zoghbi, 2003). RTT is an X-linked dominant disorder that primarily affects females and is caused by mutations in the X-linked gene *MECP2*, the methyl CpG binding protein 2 (Amir et al., 1999). Interestingly, *MECP2* mutations have also been reported in other neurological conditions including autism and Angelman syndrome (Hammer et al., 2002), and expression abnormalities occur at a high frequency in autistic brain samples without *MECP2* mutations (Samaco et al., 2004; Nagarajan et al., 2006).

MeCP2 was originally characterized based on the ability to bind methylated DNA and was proposed to repress gene expression through interactions with cofactors such as histone deacetylases (Nan et al., 1996, 1998). Gene expression profiling of *Mecp2*-deficient mouse models, however, failed to uncover clear MeCP2 target genes (Tudor et al., 2002). Several potential target genes, such as *Bdnf*, *Dlx5*, and *ID1-4*, have been identified (Horike et al., 2005; Chang et al., 2006; Peddada et al., 2006), although the dysregulation of these genes is not consistent with MeCP2 functioning as a simple transcriptional repressor. Furthermore, a recent study revealed that MeCP2 is frequently bound to the promoters of active genes and is found often in intergenic regions. This data has led to the hypothesis that MeCP2 functions as a long-range modulator of gene expression (Yasui et al., 2007).

Chromosome 15q11-13 Duplication Syndrome

Duplications of the proximal region of chromosome 15q exist as interstitial duplications and as supernumerary marker chromosomes (SMCs). The phenotypic outcome varies depending on the amount of genomic material that is duplicated and the parental origin of the aberrant chromosome. 15q duplications including the Prader–Willi/Angelman critical regions (PWACR) (breakpoints 2–3 in Fig. 2) often lead to severe mental retardation and developmental delay while less extensive duplications of centromeric and repetitive sequences result in mild to normal phenotypes (Crolla et al., 1995; Browne et al., 1997). Clinical reports of individuals with 15q duplications including the PWACR have described hypotonia, moderate to severe developmental delay, mental retardation, autistic behavior, and epilepsy as the most common phenotypes (Battaglia, 2005). Familial cases of 15q duplications have revealed that severe phenotypic manifestations are dependent on maternal inheritance since paternal inheritance of the same abnormal chromosomes has little to no phenotypic consequence (Cook et al., 1997; Schroer et al., 1998; Bolton et al., 2001).

Maternal 15q11-13 duplications are the most common cytogenetic cause of autism, occurring in approximately 1–3% of autistic individuals (Schroer et al., 1998). While the presence of autistic behaviors is very frequent in individuals with 15q duplications, the duplication is not always sufficient for the diagnosis of classic autism and often results in atypical autism (Rineer et al., 1998; Bolton et al., 2001). In addition to variable expressivity in behavioral phenotypes, the degree of mental impairment and seizures is also variable in individuals with similar duplications suggesting that genetic modifiers may exist (Borgatti et al., 2001). Gene expression profiling of lymphoblastoid cells from 15q11-13 duplication samples has revealed consistent overexpression of *UBE3A* as well as a few other nonimprinted 15q11-13 genes (Baron et al., 2006; Nishimura et al., 2007); however, this approach has failed to detect expression of brain-specific genes such as the 15q11-13 GABR genes. Therefore, it remains to be seen if differences in expression of the GABR genes may

help to explain the variability in phenotypes observed in individuals with maternal 15q11-13 duplications.

Genetic Evidence for 15q11-13 Involvement in Autism

Linkage and Association Studies in Autism

Autism is a complex neurodevelopmental disorder characterized by deficits in social interaction, language, and restricted interests/repetitive behaviors. Although a diagnosis of classic autism requires deficits in all three areas, autism spectrum disorders include a range of pervasive developmental disorders with varying degrees of impairments. Studies of the occurrence of autism spectrum disorders in families and concordance rates in twins suggest that there is a strong genetic component to autism likely involving multiple genes interacting with one another (Veenstra-VanderWeele and Cook, 2004). Many genome-wide linkage scans have been performed over the past decade, however, no single genomic region has been consistently identified to contribute to autism (Freitag, 2007). Given the challenge of identifying new loci involved in autism susceptibility, many genetic studies have focused on the well-established autism candidate locus in 15q11-13.

To date over a dozen linkage and association studies have been completed using 15q11-13 markers. While some reports have failed to find evidence for autism susceptibility in 15q11-13 (Maestrini et al., 1999; Salmon et al., 1999), many studies have found significant linkage and association (Cook et al., 1998; Bass et al., 2000; Martin et al., 2000; Buxbaum et al., 2002; McCauley et al., 2004; Curran et al., 2006). Interestingly, one difference between conflicting early studies was the use of simplex verses multiplex families, with simplex families being more likely to have significant linkage in 15q11-13 (Cook et al., 1998; Maestrini et al., 1999; Salmon et al., 1999; Martin et al., 2000). Later studies used the related endophenotypes of savant skills (Nurmi et al., 2003a) and "insistence on sameness" (Shao et al., 2003) to identify subgroups of autism families that are more likely to have autism susceptibility alleles in 15q11-13. Although a few reports found linkage in maternally expressed genes (Nurmi et al., 2001; Nurmi et al., 2003b) most significant markers are within *GABRB3* (Cook, Jr. et al., 1998; Martin et al., 2000; Buxbaum et al., 2002; Nurmi et al., 2003a; Shao et al., 2003; McCauley et al., 2004; Curran et al., 2006), making this the strongest 15q11-13 candidate gene in idiopathic autism.

GABRB3 Protein Defects in Autism Post-mortem Brain

While *GABRB3* is a clear positional and functional candidate gene for autism, mutations in this gene have not been reported in autistic individuals. To explore the possibility that this protein is involved in the etiology of idiopathic autism,

Samaco et al. (2005) quantified GABRB3 protein levels in post-mortem cerebral cortex samples from multiple autism spectrum disorders including classic autism, Rett syndrome, and Angelman syndrome. As expected, significantly reduced levels of GABRB3 protein were found in Angelman syndrome samples with maternal 15q11-13 deletions. Profound protein defects were also observed in 56% (five out of nine) of autism brain samples, suggesting that GABRB3 is commonly dysregulated in idiopathic autism. Interestingly, Rett syndrome brain samples and *Mecp2*-deficient mice showed significant ~50% reduced levels of GABRB3 protein and transcript, suggesting that MeCP2 is involved in positively regulating *GABRB3*.

Phenotypic Consequences of GABRB3 Deficiency

Mouse knockout models of *Gabrb3* have demonstrated that this receptor protein is important for both development and proper neurological function. *Gabrb3*-targeted knockout mice as well as mice with a large deletion spanning the entire β3, α5, γ3 cluster exhibit ~90% neonatal lethality, primarily due to cleft palate, and severe neurological deficits (Culiat et al., 1994; Homanics et al., 1997). Mice with deletions of the α5 and γ3 subunits or the γ3 subunit alone do not have cleft palate and appear phenotypically normal, suggesting that these subunits play a less critical role in neurological function (Culiat et al., 1994). Careful examination of surviving *Gabrb3* knockout mice has revealed abnormal behaviors such as hyperactivity (including stereotypical running in tight circles), hypersensitivity to touch and sensory stimuli, seizures, learning and memory deficits, social impairments, and deficits in exploratory behavior (Homanics et al., 1997; DeLorey et al., 1998, 2008). Interestingly, deficits in exploring novel objects and social impairments attributed to attenuated interest in unfamiliar mice are reminiscent of the "insistence on sameness" endophenotype used to find significant linkage to *GABRB3* in autism pedigrees (Shao et al., 2003).

Complex Regulation of 15q11-13 $GABA_A$ Receptors

Paternal Expression Bias of 15q11-13 $GABA_A$ Receptor Genes

Close proximity to imprinted genes and apparent paternal expression bias has led to the speculation that the 15q11-13 $GABA_A$ receptor genes are also imprinted. The mouse orthologues of *GABRB3*, *GABRA5*, and *GABRG3* are not imprinted (Nicholls et al., 1993; Buettner et al., 2004); however, several studies have found evidence for paternal expression bias of one or more of the three 15q11-13 GABA receptor subunit genes in humans. Using a technique to transfer a single paternal or maternal human chromosome 15 into mouse cell lines, Meguro et al. (1997) saw expression of *GABRB3*, *GABRA5*, and *GABRG3* from the paternally derived chromosome 15 but not from the maternally derived chromosome. Consistent with this result, Bittel

et al. (2003, 2005) performed gene expression profiling with lymphoblastoid cell lines from PWS and AS patients with UPD and 15q11-13 deletions and found much higher paternal chromosome expression of *GABRB3* and *GABRA5*. Similarly, quantification of *GABRB3* transcript in cerebral cortex from PWS UPD and deletion and AS deletion samples demonstrated that the paternal chromosome has more abundant expression when the normal biparental chromosome contribution is absent (Hogart et al., 2007).

Aberrant Monoallelic Expression of 15q11-13 $GABA_A$ Receptor Subunits in Autism

Since the 15q11-13 $GABA_A$ receptor subunit genes are implicated in multiple neurodevelopmental disorders, including idiopathic autism, imprinting analysis was performed to better understand how these genes contribute to disease. Although a bias in paternal expression was observed with abnormal chromosome contributions (Meguro et al., 1997; Bittel et al., 2003, 2005), equal parental expression (biallelic expression) was observed in 21 control cerebral cortex samples, demonstrating that the 15q11-13 $GABA_A$ receptor subunit genes are not normally imprinted (Hogart et al., 2007). Imprinting analysis of autism and Rett syndrome cerebral cortex samples revealed that four out of eight autism samples and one out of five Rett samples had monoallelic expression of one or more $GABA_A$ receptor subunit gene (Hogart et al., 2007). DNA sequencing of the coding and regulatory regions did not uncover mutations that could explain the aberrant monoallelic expression. Parental identity of the expressed allele could not be determined; therefore the mechanism underlying the aberrant monoallelic expression could not be elucidated. Interestingly, autism samples with monoallelic expression of any one of the three $GABA_A$ receptor genes had significantly reduced GABRB3 protein levels, suggesting that monoallelic expression of 15q11-13 GABR genes predicts a subset of autism samples with dysregulation of the GABAergic system (Samaco et al., 2005; Hogart et al., 2007).

Homologous Pairing of 15q11-13 Chromosomes in Neurons

Homologous chromosome interactions are essential for proper chromosomal segregation during meiosis and mitosis but increasing evidence suggests that physical associations between chromosomes are also important for establishing proper gene expression. Transient pairing of homologous X chromosomes during development is essential for proper X chromosome inactivation in females (Xu et al., 2006). Physical interactions between nonhomologous chromosomes are implicated in coordinating expression of alternatively expressed genes in different T cell fates (Spilianakis et al., 2005). In addition to aiding in silencing, chromosome pairing has also been shown to positively influence expression of associated alleles by colocalizing genes to shared sites of active transcription called transcription factories (Osborne et al., 2004; Ling et al., 2006).

Homologous pairing of 15q11-13 alleles has been observed in late S phase in lymphocytes as well as nondividing nuclei of mature neurons (LaSalle and Lalande, 1996; Thatcher et al., 2005). Homologous pairing of 15q11-13 alleles in neurons is a developmentally regulated process that is partially dependent on the binding of MeCP2, the methylated CpG binding protein 2 (Thatcher et al., 2005). Chromatin immunoprecipitation experiments have demonstrated that MeCP2 binds throughout 15q11-13 (Yasui et al., 2007); however, in the absence of MeCP2, proper imprinted expression of 15q11-13 genes is maintained (Balmer et al., 2002). Interestingly, significantly reduced expression of *GABRB3*, a nonimprinted gene, was observed in *Mecp2*-deficient mice and Rett syndrome brain samples (Samaco et al., 2005; Hogart et al., 2007). These observations have led to the hypothesis that homologous pairing of 15q11-13 alleles mediated by MeCP2 is required for optimal expression of *GABRB3*.

Rett syndrome and autism brain samples with MeCP2 expression defects are deficient in pairing of 15q11-13 *GABRB3* alleles in neurons (Thatcher et al., 2005). Homologous pairing of 15q11-13 alleles may be important for obtaining optimal *GABRB3* expression because this locus is subject to imprinting and therefore paternal and maternal chromosomes do not have equal propensities for gene expression. The paternal 15q11-13 chromosome has many more actively expressed genes and we hypothesize that this more active chromatin state leads to the paternal expression bias that has been previously reported for *GABRB3* (Bittel et al., 2003, 2005). Although we were unable to determine the parental allele that was expressed in autism samples with monoallelic GABR expression (Hogart et al., 2007), we speculate that the maternal allele was silent due to deficiencies in pairing. Interestingly, although a clear connection has been established between 15q11-13 pairing, GABRB3 expression, and MeCP2, reduced GABRB3 expression in Rett syndrome patients did not always coincide with loss of biallelic GABR expression. This discrepancy suggests that additional defects such as DNA methylation or other epigenetic abnormalities such as histone modifications may contribute to the regulation of 15q11-13 GABR genes and the pathology of autism.

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Cholinergic Component of Autism Spectrum Disorder

Elizabeta B. Mukaetova-Ladinska, Jodie Westwood and Elaine K. Perry

Introduction

The characteristic clinical phenotype of autism is associated with developmental delays in multiple areas of cognitive and behavioural functioning, including disturbed sensorimotor and perceptual performance and abnormalities/impairment in attentional processes and motivation. The degree of the characteristic clinical symptoms varies significantly, from profoundly mentally retarded to high-functioning Asperger's syndrome individuals. Autism incidence rates are currently predicted as 1:150 births (CDCP, 2007), with a threefold higher prevalence in males. The diversity of the variety and severity of symptoms associated with autism contributes to difficulties in studying this disorder. Comorbidities are frequently reported in autism (e.g. obsessive compulsive disorder, epilepsy, Tourette syndrome and attention deficit hyperactivity disorder; Gillberg and Billstedt, 2000). Furthermore, the use of different diagnostic criteria limits the continuity between studies.

Abnormal Brain Development in Autism

A variety of changes in brain volume and function, as demonstrated by (functional) MRI and PET, have been demonstrated in autism. These changes appear to be age dependent and being more pronounced in younger autistic children (<3–12.5 years; Courchesne et al., 2001, Mills Schumann et al., 2004). One of the most consistent findings is the cerebral overgrowth (Fig. 1), which may be the result either of white matter (Herbert et al., 2004) and/or of grey matter (Palmen et al., 2004) hypertrophy. Similarly, the cortical folding (as measured via the gyrification index) appears to decrease with ageing in the autistic subjects (Hardan et al., 2004).

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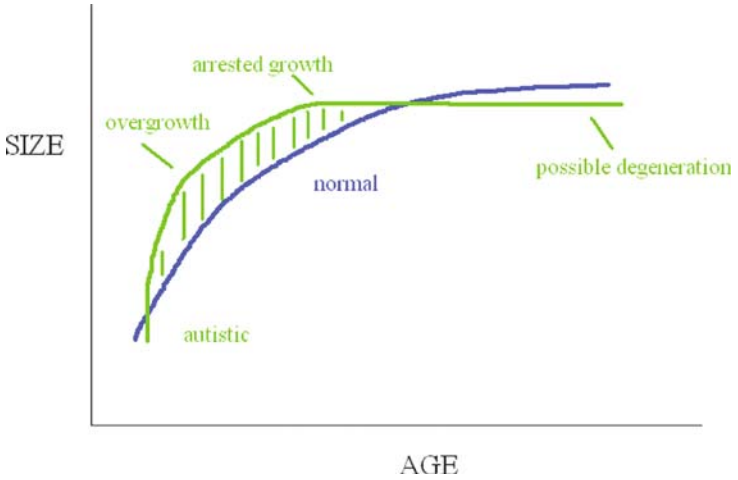

Fig. 1 Model of early brain overgrowth followed by arrested growth and degeneration. The *blue line* represents a typically developing person and the *green line* a person with autism (adapted from Courchesne et al., 2007)

The possible model for brain growth for people with autism spectrum disorder consists of a period of overgrowth before the age of 5 years, in which several regions of the brain (and overall brain size) are enlarged. From 5 years onwards, this period of growth stops. In some autistic individuals this may be followed by a period of degeneration – a decrease in neuron numbers and volume reduction in some areas of the brain (Courchesne et al., 2007), as reported in various post-mortem studies.

The reasons for the brain overgrowth in autism are unknown, and it may be attributed to alterations in neuroregulatory proteins and neurotransmitters, as well as inflammation. The maternal inflammation may play a role in the autistic child brain overgrowth. Thus, the activation of foetal microglia gives rise to cholinergic neurons projecting to medial temporal lobe and various cortical areas, resulting in excessive numbers of cholinergic neurons and six to eightfold higher levels of choline acetyltransferase (Acevado et al., 2007). Furthermore, the levels of various growth-related hormones, including insulin-like growth factors (IGF-1 and IGF-2), insulin-like growth binding protein IGFB-3 and growth hormone binding protein (GHBP) are significantly higher in autistic children than in age-matched control subjects (Mills et al., 2007).

One of the earliest neuroregulatory systems to occur in the developing brain is the nicotinic acetylcholine receptors (nAchRs) that start being established as early as the first half of the first trimester and are followed by development of dopamine and norepinephrine neurons in the following 7 weeks, whereas the cholinergic fibres start entering the brain cortex form 28–40 weeks of gestation. Furthermore, transient increase in regional patterns of various nAChR subunits during pre- and postnatal development occur during periods of neuronal development, including neurogenesis, migration, differentiation and synaptogenesis (reviewed in Dwyer et al., 2008).

In addition to the latter, nAchR may mediate neuronal pruning and regulate neural pattern formation by decreasing overproduction of cells and outgrowth and guiding remaining neuritis to their targets. Since nAChRs are reduced in autism, their underactivity could result in brain overgrowth.

The Excitatory/Inhibitory Ratio Theory

The autistic clinical phenotype may be a consequence of the enlarged brain containing excess neurons. If this is the case, then the latter will result in an excess of excitatory neurons, causing an imbalance to the excitatory/inhibitory impulse ratio in a particular neural circuit. This neural circuit with increased excitatory impulses, due to the hyperexcitability preventing correct functional differentiation of the neurons or a lack of pruning (Rubenstein and Merzenich, 2003), will result in being unstable and 'noisy'. This hypothesis has been confirmed in the mutant mouse model of autism which contains excess olivocerebellar climbing fibres due to ineffective pruning in early development leading to Purkinje cell loss (Mariani, 1982), similar to the Purkinje cell decrease present in autistics (Fatemi et al., 2002a). Since neocortical areas in autism are also affected by overgrowth, the neural circuits responsible for social interactions and memory may be affected in this way, thus resulting in the characteristic clinical phenotype of autism.

Acetylcholine influences attention, short-term memory, and sleep–waking cycle through modulatory influence on cortical neurons (reviewed in Pepeu and Giovannini, 2004). It has been proposed that the behavioural changes mediated by the acetylcholine result from its selective effects on the intrinsic membrane properties of cortical inhibitory intraneurons (Levy et al., 2008). Acetylcholine has been shown to reduce the strength of excitatory (glutamatergic) synapses. An increase in excitatory activity could be caused by genetic defects in the glutamate signalling pathway. Association between polymorphisms in the glutamate 6 receptor gene (chromosomal location 6q21) and autism was found in a recent study (Jamain et al., 2002). This finding was replicated in a family-based association study with Chinese Han trios (two parents with one affected child) (Shuang et al., 2004). A recent study demonstrated the effect of acetylcholine on local cortical neurons necessary for shaping sensory processing via reduction of local excitatory input to inhibitory neurons by acetylcholine (Levy et al., 2008).

Cholinergic System

The cholinergic system in mammalian brain is anatomically diverse, including basal forebrain projections to cerebral cortex and hippocampus, brainstem nuclear projections to the thalamus and cerebellum among other targets. The projections to the thalamus from the brainstem are massive (up to 90% of all brainstem input) and are

involved in arousal including changes in the sleep/wake cycle. The forebrain projections are associated with maintenance of cognitive abilities, including memory and learning.

Acetylcholine (Fig. 2) released from growing axons regulates growth, differentiation and plasticity of the developing central nervous system (Lauder and Schambra, 1999) and modulates neurite outgrowth in developing neurons (Tata et al., 2003). Brain-derived neurotrophic factor (BDNF) encourages the growth of developing neurons and maintains mature neurons. Rats with neonatal basal forebrain cholinergic lesions have impaired cognitive function (Hohmann and Berger-Sweeney, 1998) and are being used as animal models for autism (Walker et al., 2007).

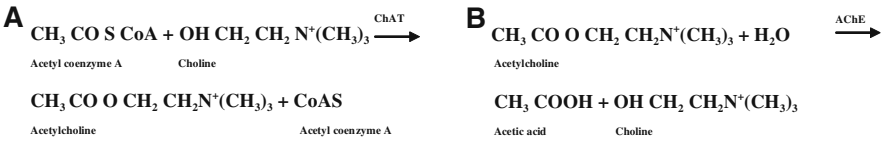

Fig. 2 Acetylcholine production and metabolism. (**A**) acetylcholine production [by choline acetyltransferase (ChAT)] and (**B**) breakdown of acetylcholine [by acetylcholinesterases (AChE)]

There are two classes of cholinergic receptors in the brain: muscarinic and nicotinic, each including a range of subtypes. Nicotinic receptors consist of different combinations of α and β subunits, 11 of which can be found in the central nervous system (α2–α10 and β2–β4; reviewed in Gotti et al., 2006, Dwyer et al., 2008). Nicotinic receptors, among many other neuronal sites, are also located on GABA neuronal dendrites (Sylvester et al., 2004) and mediate GABA release, and nicotinic agonists modulate dendritic growth (Torrao et al., 2003).

The nicotinic receptors are involved in regulating neuronal development, including neurogenesis, migration, differentiation and synaptogenesis. Besides their role in pre- and perinatal circuit formation, they play an important role in age-related cell degeneration. The exact nAChR subtype composition in different brain parts is still not complete, and this is largely due to sensitivity of different methodological approaches that have been used, and this is further complicated since specific nAChR subunits can differ between species. Thus α2β2 receptor although expressed in the primate and human cortex is absent in the respective areas of the rodent brain. This is in contrast to the abundance of α4β2 receptors present in the rodent brain. Similarly, for many of the possible subtypes there is no evidence that they are expressed in the mammalian brain, whereas other subtypes are expressed only in selected brain parts. Thus α3β2 subunits are expressed in the visual pathway, α4α6β2β3 receptor subtypes in visual and mesostriatal pathways, and most likely having a specific role in the brain function (reviewed in Steinlein and Bertrand, 2008). The α7 nAChR has neuroprotective function and is highly expressed on hippocampal neurons and cholinergic projection neurons from the basal forebrain. Interestingly, the allosteric potentiation of α7 nAChR may mediate the antipsychotic-like effect of the galantamine adjunctive treatment (Wiker et al., 2008), thus providing further support for development of novel α7nAChR

selective antipsychotic treatment that can be used in schizophrenia and other related psychiatric disorders.

The α4β2 nAChR is the predominant heteromeric receptor in the brain, binds nicotine and other nicotinic agonists, e.g. cytisine and epibatidine. As a principal nicotinic AChR is more ubiquitous and is predominant in cortex, striatum, superior colliculus, lateral geniculate nucleus and cerebellum. When activated it facilitates excitatory inputs and is present very early in the developing brain in various brain regions.

The muscarinic acetylcholine receptors (mAChR) mediate most of the action of acetylcholine in the CNS and peripheral nervous system, as well as in the end organs of the parasympathetic nerves, e.g. cardiac and smooth muscles and secretory glands. In mammals, five mAChR (M1–M5) have been identified, with each receptor subtype being the product of a different gene. mAChRs are implicated in learning and memory. M1 and M3 mAChRs are most commonly postsynaptic, whereas M2, M3 and M4 are located presynaptically. M1 and M2 mAChR are predominant in the cerebral cortex and hippocampus and may play a role in cognitive processing, e.g. working memory. M2 receptors are found both on cholinergic and on noncholinergic terminals (reviewed in Nathanson, 2008).

Post-mortem Studies

Muscarinic Receptor Changes in Autism

Although muscarinic receptor changes have not been extensively investigated in autism, the findings are very similar to that of the nicotinic receptors. Thus, M1 receptor binding is decreased by 30% in the cortical regions of autistic subjects (Perry et al., 2001). This could be attributed to epilepsy, as 40% of autistic children are estimated to suffer from epilepsy (Minshew et al., 1997). Furthermore, low numbers of M1 receptors have been reported in hippocampal sclerosis, linked with temporal lobe epilepsy (Pennell et al., 1999). However, the findings of Perry et al., (2001) were not linked to epilepsy within the series of autism individuals examined. The findings from the latter study are similar to those reported for schizophrenia, where one of the more consistent neurotransmitter abnormalities is loss of the various muscarinic receptors in different areas (Deng and Huang 2005).

Nicotinic Receptor Changes in Autism

The cholinergic system in autism has been investigated in a number of post-mortem studies (Table 1). In the parietal and frontal cortices of autistic adult subjects, levels of nicotinic receptors were 65–73% lower than in controls (Perry et al., 2001). In the parietal cortex specifically, there were lower levels of α4 and β2 nicotinic receptor subunits, the high-affinity-type nicotinic receptor (Fig. 4). These abnormalities

Table 1 Summary of post-mortem studies focusing on the cholinergic system

Study	Subjects	Main findings
Perry et al. (2001)	Seven autistic adults (diagnosed by DSM-IV criteria), six MR adults, three adults with Down's syndrome and six controls.	In comparison to controls, autistic subjects have lower cortical M1 receptor binding by 30%, lower level of nicotinic receptors by 65–73% in the parietal and frontal cortices, lower levels of α4 and β2 nicotinic receptor subunits in the parietal cortex and threefold higher levels of BDNF in the basal forebrain
Lee et al. (2002)	Eight autistic MR adults (diagnosed by DSM-IV criteria), 11 non-autistic MR adults and 10 controls.	Autistic subjects have a reduction of 40–50% nicotinic receptor binding to the agonist epibatidine (subunits α3, α4 and β2) in the granule cell, Purkinje and molecular layers. This is accompanied by a threefold increase in the nicotinic receptor binding α-bungarotoxin (α7 subunit) in the granule cell layer, a decrease in α4 receptor subunits in Purkinje and other cell layers, and a nonsignificant increase in α7 subunit in the granule cell layer
Martin-Ruiz et al. (2004)	Six autistic adults (diagnosed with DSM-IV criteria), eight controls	In the parietal cortices of autistic subjects, α4 subunit mRNA levels, protein expression and receptor binding density were lower than controls, whereas β2 subunit protein expression was lower. In the cerebellum they had higher levels of α4 subunit mRNA and non-significant increase in α7 subunit mRNA. These changes were accompanied by a significant increase in receptor binding density, whereas the protein expression and receptor density were decreased
Ray et al. (2005)	Three autistic adults (diagnosed by DSM-IV criteria), three controls	Autistic subjects have decreased levels of α7 and β2 immunoreactive neurons in the paraventricular nucleus (PV) and nucleus reunions. There was no difference in the co-expression of α7 and glutamic acid decarboxylase in the PV between autistic and control subjects, suggesting that the loss of α7 subunits in autism is not due to a loss of GABAergic neurons

DSM-IV: diagnostic and statistical manual of mental disorders; MR, mentally retarded; M1, muscarinic M; BDNF, brain-derived neurotrophic factor; PV, paraventricular nucleus.

could be caused by abnormal cortical neuronal morphology (for example, synaptic and dendritic abnormalities, Mukaetova-Ladinska et al., 2004), similar to other developmental disorders (such as schizophrenia in which nicotinic receptors have also been implicated).

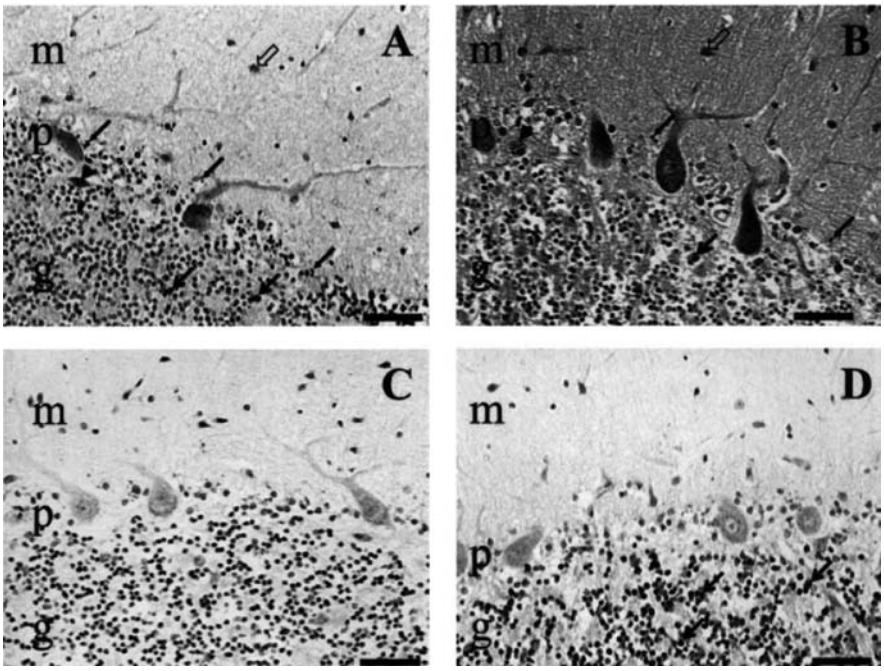

Fig. 3 nAChR subunit immunochemistry in the cerebellar cortex. nAChR (**A** and **C**) α4 and (**B** and **D**) α7 subunits in (**C** and **D**) autistic compared to (**A** and **B**) control. M, molecular layer; p, Purkinje cell layer; g, granule cell layer

In the cerebellar cortex, nicotinic receptors binding the agonist epibatidine (α3, α4 and β2) were 40–50% lower in the granule cell, Purkinje and molecular layers in autistic subjects than controls (Fig. 3). In the granule cell layer, there was a threefold increase in the nicotinic receptor binding the agonist α-bungarotoxin (α7 subunit) (Lee et al., 2002). This result in particular should be highlighted, as the gene which encodes the α7 subunit is found near to q11–15 on chromosome 15 (a region implicated in autism, Lamb et al., 2000). In contrast, choline acetyltransferase (ChAT) levels were similar in autistic and control subjects (Lee et al., 2002). This suggests that the presynaptic cholinergic system is not altered in autistics.

These substantial differences in nicotinic receptors between autistic subjects and controls led to a study investigating the levels of mRNA, protein expression and receptor binding densities of the various nicotinic receptor subunits (Fig. 3). In the parietal cortices of autistic subjects, α4 mRNA levels, protein expression and receptor binding densities were all lower than in controls (Martin-Ruiz et a., 2004). β2 protein expression was also found to be lower. Although the cerebellar α4 mRNA levels increased, the protein expression and receptor binding densities decreased. Levels of α7 receptor subunit binding density were also increased in the cerebellum (as well as non-significant increases in mRNA levels and protein expression; Martin-Ruiz et al., 2004). These cholinergic system abnormalities could be related

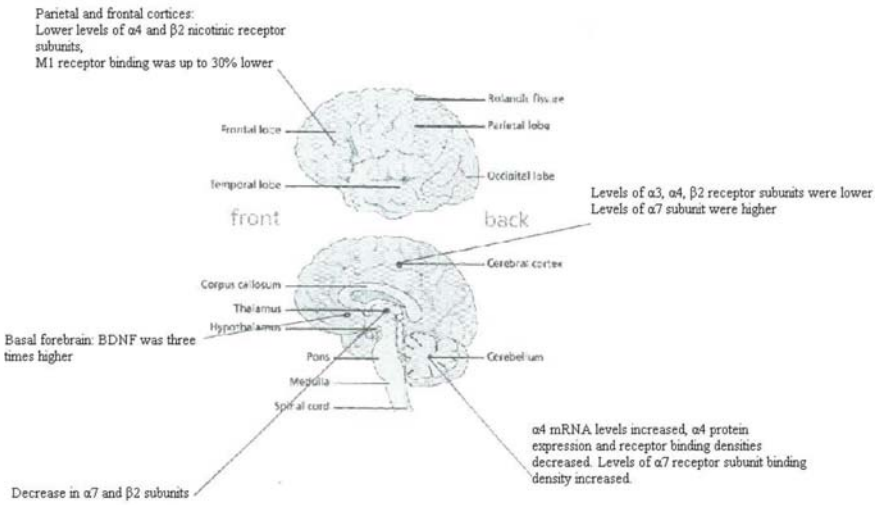

Fig. 4 Location of cholinergic deficits in the autistic brain (BDNF: brain-derived neurotrophic factor)

to cortical cell loss or dysfunction, supported by evidence from other studies that protein expression of Bcl2 (an antiapoptopic protein) is lower in ratio to p53 (an apoptotic regulator) in this area, possibly promoting apoptosis (Fatemi and Halt, 2001, Fatemi et al., 2001).

In the thalamus, nicotinic receptor subunits were decreased in the numbers of α7 and β2 immunoreactive neurons in the paraventricular nucleus (PV) and nucleus reuniens (NR) in autistic subjects (Ray et al., 2005; Fig. 4). These deficits in nicotinic receptor subunits perhaps result in the failure of the PV and NR (and so the thalamus) to correctly modulate sensory input. This effect would then be transferred throughout the limbic system to other parts of the brain, contributing to the incorrect sensory processing found in autism (Martin-Ruiz et al., 2004). The co-expression of α7 and glutamic acid decarboxylase (GAD) study indicated that despite the decrease in α7, the expression of GAD in the PV was similar in both autistic and control subjects (Ray et al., 2005). This suggests that the decrease in α7 subunits in the thalamus is not associated with the GABAergic system.

Growth Factors Modulating the Cholinergic System

Neurotrophins mediate acetylcholine release from cholinergic neurons (Huh et al., 2008). Brain-derived nerve growth factor (BDNF) is a member of the nerve growth factor family (neurotrophins) that contributes to both pre- and postnatal brain development. BDNF supports the neuronal survival, maintains neuronal activity and plasticity, modulates neurotransmitter release and mediates long-term potentiation

and memory fixation. Since BDNF influences nAChR activity (Fernandes et al., 2008) it may well be associated with impaired brain function in a number of diseases characteristic with alterations in cognition. Thus, BDNF expression has been reported to be three times as high as control subjects in the basal forebrain of autistic individuals. This could be part of autism symptomology, as one recent report studying autistic babies found 62 out of 64 to have increased levels of BDNF, calcitonin gene-related peptide and vasoactive intestinal peptide (Nelson et al., 2000).

Brain BDNF expression parallels the age-related changes seen in serum BDNF levels (Karege et al., 2002), and the latter can be a useful tool to explore the brain development changes in the autistic spectrum. Serum BDNF is detected in humans and primates and has been investigated in various psychiatric diseases, e.g. depression, schizophrenia and eating disorders that all have lower levels of serum BDNF than the control subjects (Toyoka et al., 2002, Nakazato et al., 2003, Shimizu et al., 2003). In control subjects, BDNF serum levels increase over the first decade and slightly decrease after reaching adult level, whereas in autism, the serum BDNF levels are significantly lower in children (0–9 years) compared to teenagers/adult autistic individuals or age-matched control subjects, whereas their level is higher in the second decade of life compared to control subjects (Katoh-Semba et al., 2007). However, these findings have not been replicated. Thus, Hashimoto et al., 2006 found a decrease in serum BDNF in autistic subjects aged 14–22 years. In autism, BDNF production is enhanced during the neonatal period (Miyazaki et al., 2004) but reduced in adult (18–26 years) subjects (Hashimoto et al., 2006). However, a more recent analyses using more refined immunoassay technology failed to replicate the BDNF finding in blood (Nelson et al., 2006). Furthermore, even young autistic children in their first decade show significantly higher levels of serum BDNF (Connolly et al., 2006). Interestingly, no changes in NGF were noted in the autopsy study (Perry et al., 2001), and further research is needed to address the brain and the peripheral BDNF content in the autistic spectrum diseases.

Interpretations

The significance of loss of nicotinic receptors in neocortical, cerebellar, thalamic and striatal regions in adult autistic individuals (Perry et al., 2001, Lee et al., 2002, Martin-Ruiz et al., 2004, Perry et al., unpublished) is unknown. Since these receptors are widely localized pre- and post-synaptically, reduced expression of nAChR (especially $\alpha 4\beta 2$ nAChR, Martin-Ruiz et al., 2004) indicates widespread nicotinic receptor dysfunction and disconnectivity. Alternatively, in distinct brain areas, cell loss may also be present and contribute to these changes, as demonstrated via altered ratio of antiapoptotic Bc12 and apoptotic regulator p53 in the parietal lobes in autism (Fatemi and Halt, 2001). Since nAChRs are known to modulate the release of other neurotransmitters, e.g. GABA and glutamate (Lavine et al., 1997, Baulac et al., 2001), the deficit of nicotinic receptors may denote the early imbalance between the excitatory (glutamatergic) and inhibitory (GABAergic) interneurons in the autistic

brain and thus would increase the excitatory/inhibitory impulse ratio further, consequently resulting in worsening of the clinical presentation of autism. To explore these possibilities immunohistochemical double labelling studies using antibodies to specific nicotinic receptor subunit(s) together with GABA or glutamate markers are needed.

The cholinergic brain pathology studies in autism, discussed above, have all been conducted on relatively small numbers of adult individuals in restricted brain areas and not related to core or other clinical symptoms. A key question is the extent to which these receptor changes are central in the disorder and whether they emerge at an early or late stage. While most neurobiological investigators tend to consider 'bottom up' mechanistic, causal explanations, it is equally possible that transmitter dysfunctions arise from 'top down', i.e. differences in psychological/physiological modes compared to normal affect the regulation of synaptic signalling and receptor levels. The findings thus far in adults are more an indication of the need to take such investigations further than a contribution to aetiopathology. Neuroimaging studies in affected individuals are feasible since there are now reliable markers for muscarinic and nicotinic PET or SPECT receptor imaging. There are recent reports of increased choline levels in autism (Hardan et al., 2008; Gabis et al., 2008, Vasconcelos et al., 2008) using MRI proton spectroscopy imaging or MR SPECT that may be relevant to cholinergic dysfunction. However, these findings are not conclusive, and similar choline levels have been described in both autistic boys aged 6–17 years and control subjects using the same methodology (DeVitto et al., 2007). Modelling in mice is considered by some to be of value despite the vast species difference in cerebral complexity. Among models of autism a β2 nicotinic receptor mutant has been reported to exhibit characteristics of the disorder (Granon et al., 2003).

Cholinergic Therapies for Autism

Acetylcholinesterase Inhibitors (ChEI)

In the light of cholinergic abnormalities apparent in adult autistic brain tissue, acetylcholinesterase inhibitors may be a useful treatment to counter one or more of the cognitive and non-cognitive symptoms of the condition. Acetylcholinesterase inhibitors (ChEI) increase the rate at which acetylcholine is broken down, so more acetylcholine is present in the synaptic cleft (Fig. 2). Donepezil, galantamine and rivastigmine are all ChEI, licenced for the treatment of Alzheimer's and also applied to a range of other neuropsychiatric disorders such as Parkinson's disease dementia or Lewy body dementia, Down syndrome, delirium, schizophrenia, depression, mania, traumatic brain injury and attention deficit hyperactivity disorder. (reviewed in Yoo et al., 2007). In the case of dementia, ChEI have been shown to have significant benefit of both cognitive and behavioural changes and improve global function as well as activities of daily living (reviewed in Farlow et al., 2008).

ChEI Treatment in Schizophrenia

Similar findings of improvement of both cognitive and non-cognitive changes have now been reported for schizophrenia. Thus, donepezil significantly improves both the positive and the negative symptoms in schizophrenic subjects (Keefe et al., 2008), depressive symptoms (Risch et al., 2006) as well as improvement in verbal learning (Erickson et al., 2005) in schizophrenia. However, benefits seem to be restricted to relatively younger schizophrenic subjects, whereas in the elderly patients with chronic schizophrenia, benefits are not seen (Mazeh et al., 2006). Similar findings of absence of benefits on neurocognition and/or social cognition were also found in stable community-treated schizophrenia patients (Freudenreich et al., 2005, Kohler et al., 2007). The latter findings have now also been reported for rivastigmine-treated schizophrenia subjects (Sharma et al., 2006). However, longer (12 months) treatment with higher doses of rivastigmine (2×6 mg/day) appears to improve cognitive function, learning and memory as well as attention and quality of life (Lenzi et al., 2003). Most recently, selective benefits in processing speed and verbal memory have been reported in galantamine-treated people with schizophrenia in a 12-week, double-blind, placebo-controlled, randomized clinical trial (Buchanan et al., 2008). However, these benefits appear to be diminished in non-smokers with schizophrenia (Dyer et al., 2008). The addition of donepezil (up to 10 mg/day) is associated with significant improvement (from 37.5 to 63.6%) of tremor following 4 weeks treatment in elderly schizophrenia patients with tardive movement disorders (Bergman et al., 2005).

The findings regarding improvement of cognitive deficits in schizophrenics with memory problems are not conclusive. A recent meta-analysis reported small to medium improvement in short- and long-term memory in schizophrenia patients compared to their baseline performance, though when compared to control (placebo-treated patients), they performed worse on both working and long-term memory (Stip et al., 2007). Furthermore, the length of treatment may also influence the outcome measures in cognitively impaired schizophrenia subjects. Thus, ChEI treatment (e.g. rivastigmine 9 mg/day) in chronic schizophrenia complicated with comorbid dementia has been found to be beneficial, as seen via the improvement of cognitive, behavioural functioning and activities of daily living (Mendelsohn et al., 2004). In contrast, most recent study (Chouiinard et al., 2007), using the same dosage of rivastigmine over 6 months, failed to replicate these findings. These findings need to be replicated on a larger number of participants and over longer periods.

ChEI Treatment in Down Syndrome (DS)

Cholinesterase inhibitors have been used widely in the treatment of both cognitive and behavioural changes in adult and elderly Down syndrome (DS) individuals. Thus, DS adults treated with 3–5 mg/day donepezil have improvement in verbal

and written communication, as well as quality of life (Kondoh et al., 2005). The improvement in verbal communication appears to be one of the consistent findings for DS subjects with no cognitive impairment (Johnson et al., 2003). DS adults with dementia treated with the ChEI (e.g. rivastigmine) appear to have slower decline in global functioning and adaptive behaviour over 24 weeks compared to the untreated group (Prasher et al., 2005).

Most recently, ChEI use has been reported for DS children. Heller et al., (2004) reported significant improvement of expressive and receptive language performance in 7 DS children (ages 8–13 years) in 16-week open-label trial (donepezil, 2.5–5 mg/day, followed by 6-week washout period). These findings were confirmed when the study was extended to 22-week open-label trial (donepezil 2.5 and 5 mg/day; Spiridigliozzi et al., 2007). Overall, donepezil was well tolerated, and subjects had language improvement, though some of the participants exhibited increased irritability and/or assertiveness. Similar results of improvement of adaptive function, attention, memory and language have been reported for rivastigmine in 11 young DS subjects (ages 10–17 years; Heller et al., 2006). These results need to be confirmed in larger controlled studies that will employ tests that will cover the performance across multiple psychological domains, including functional ability of the DS subjects.

ChEI Treatment in Autism

Donepezil: Studies conducted with donepezil in rodents and primates are summarized in Table 2. Donepezil inhibits acetylcholinesterase activity and improves memory and spatial learning. Studies in primates mirror these results. There are few studies assessing the value of treating autistics with donepezil (Table 3). Hardan and Handen (2002) studied the effects of donepezil on eight autistic children and adolescents, aged 7–19 years. All of the patients were diagnosed using DSM-IV criteria and were openly treated with donepezil. About 50% of patients showed improvements [as assessed by the aberrant behaviour checklist (ABC) and the Clinical Global Impression Scale] in irritability and hyperactivity. No other changes were observed, but cognition and memory tests were not conducted. Only two side effects were reported: one patient had gastrointestinal problems and another had a mild increase in irritability. Chez et al., (2003) conducted a double-blind study with donepezil with 43 children with autism or pervasive developmental disorder. For 6 weeks randomized groups were administered either donepezil or placebo, followed by a 6-week open trial with donepezil. After 6 weeks treatment with donepezil, improvements in autistic symptoms were reported using the childhood autism rating scale (CARS) scoring system, and improvements were seen in receptive and expressive language scores. When donepezil was compared to the placebo, there were significant improvements seen with the active drug.

Table 2 Animal trials with donepezil

Species	Study	Design/procedure/variable	Dependent variable	Main findings
Rodents	Luine et al. (2002)	Five rats in each group: control administered saline, chromaproline (270 μg/kg/day), chromaperidone (279 μg/kg/day) and donepezil (1 mg/kg/day)	Visual recognition memory task, spatial memory task, 6-min open field (e.g. grid crossing)	Rats administered with donepezil showed improvement in the tasks. Chromaproline rats showed improvement in spatial memory after 3 weeks of administration.
	Dong et al. (2005)	Thirty-two control mice and Tg2576 mouse model of AD were compared over a 6-week period with administration of donepezil (0.1, 0.3 and 1.0 mg/kg). Physostigmine and saline also compared	Performance on spatial reversal learning, spatial learning, fear conditioning, ambulation and foot shock sensitivity	Decrease in the number of trials to succeed at spatial learning task, reversal learning. Increase in fear conditioning task
	Prickaerts et al. (2005)	Twenty-four rats were administered donepezil (0.1, 0.3 and 1.0 mg/kg) before and after learning first trial. Metrifonate and sildenafil also compared	Performance on an object recognition task	Improvements only seen when donepezil was administered before the task, at the highest dose.

Table 2 (continued)

Species	Study	Design/procedure/variable	Dependent variable	Main findings
	Hayslett and Tizabi (2005)	Male ICR mice treated with donepezil (2×0.1 mg/kg), nicotine (2×0.5 mg/kg) and haloperidol (0.4 mg/kg) for 14 days	DOI-induced head twitch response	Donepezil significantly increased 5-HT2A mRNA level, but not the receptor density in striatum. Cortex had significantly reduced 5-HT2A receptor density. Anti-tic properties of donepezil related to antagonism of cortical 5-HT2A receptors
	Csernansky et al. (2005)	Mice were administered MK-801 (0.05–0.1 mg/kg) 30 min after donepezil (0.10, 0.30 or 1.00 mg/kg)	Behavioural tests to assess spatial learning, locomotion, fear conditioning and shock sensitivity	Galantamine did not ameliorate MK-801-induced deficits in spatial reversal learning and in contextual and cued memory in dose-dependent manner. Similarly, it did not reverse MK-801-induced hyperlocomotion. However, it altered shock sensitivity

Table 2 (continued)

Species	Study	Design/procedure/variable	Dependent variable	Main findings
	Hohnadel et al. (2007)	Fifty rats administered with 0.3 mg/kg scopolamine, 0.5 mg/kg apomorphine or 0.1 mg/kg MK801 10 min prior treatment with 1 mg/kg donepezil	Prepulse inhibition (PPI) test	Donepezil ameliorated PPI deficits induced by scopolamine and apomorphine, but not effective in the MK801 model.
	Rueda et al. (2008)	Ts65Dn mice treated with donepezil and non-competitive GABA(A) antagonist pentylenetetrazole (PTZ) for 8 weeks	Sensorimotor abilities (e.g. vision, hearing, strength and motor coordination) and locomotor activity assessed	Donepezil did not modify learning and memory in both Ts65Dn and control groups. However, PTZ rescued Ts65Dn performance in the Morris water maze.
Primates	Rupniak et al. (1997)	Nine male rhesus monkeys, each animal received placebo, 0.003–0.06 mg/kg donepezil, with at least 1 drug-free day	Performance on spatial and visual recognition tasks	Improvements in accuracy at 0.03 and 0.05 mg/kg donepezil.

Table 2 (continued)

Species	Study	Design/procedure/variable	Dependent variable	Main findings
	Tsukada et al. (2004)	Five younger (mean = 5.2 years) and five aged (20.3 years) male rhesus monkeys administered with donepezil (50 or 250 μg/kg) or saline	PET scan 45 min after administration of donepezil or saline. Performance on oculomotor delayed response task and visually guided saccade task 30 min after administration of donepezil or saline	Dose-dependent increases in acetylcholine were seen in the frontal cortex of the young monkeys, and to a lesser extent in the aged monkeys. Dose-dependent improvements were seen in working memory for the aged monkeys.

AD, Alzheimer's disease; PET, positron emission tomography. Ts65Dn mice are trisomic for most of the MMU16 region homologous to HSA21. This mouse model shares many phenotypic characteristics with people with Down syndrome, including behavioural and cognitive changes.

Table 3 Ch EI trials in autism

ChEI	Study	Trial/subjects	Criteria for diagnosis	Main findings
Donepezil	Hardan and Handen (2002)	Eight autistic children and adolescents (7–19 years) Open trial	DSM-IV	50% of subjects had improvement on ABC and Clinical Global Impression Scale.
	Chez et al. (2003)	Forty three children with autism or pervasive developmental disorder. Double-blind study over first 6 weeks, followed by open-labelled 6-week study	DSM-IV	Improvements in receptive and expressive language scores, using CARS.
	Hetrzman (2003)	One adult treated with 5 mg/day donepezil	DSM-IV	Verbal and behavioural regression after 1-month treatment.
Rivastigmine	Chez et al. (2004)	Open-label study. Thirty two children (unspecified age)	DSM-IV	Improvements in autistic behaviour, particularly verbalization. Assessments done using Childhood Autistic Rating Scale, Gardner's Expressive and Receptive One-Word Picture Vocabulary tests and the Conners Parent Rating Scale.
Galantamine	Niederhofer et al. (2002)	Twenty boys (mean age 7.4 years). Dose and duration of treatment not specified.	ICD-10	Improvements in hyperactivity, inadequate eye contact, and inappropriate speech. ABC used.

Table 3 (continued)

ChEI	Study	Trial/subjects	Criteria for diagnosis	Main findings
	Hertzman (2003)	Three autistic adults treated with 4 mg/day galantamine, the dose increased to 12 mg/day after 2 months.	DSM-IV	Improvement in expressive language and communication.
	Nicolson et al. (2006)	Thirteen children (mean age 8.8 years). 12-week open-label trial	DSM-IV	61.5% children responded. Improvements in irritability, social withdrawal, emotional lability, inattention, and aggression. ABC, Conners Parent Rating Scale-Revised, Children's Psychiatric Rating Scale and Clinical Global Impressions Scale used.

Although these two studies suggest that donepezil may be effective in treating children and adolescents with autism, the results are not conclusive and more double-blind placebo studies need to be carried out.

Galantamine: Galantamine in addition to AChE inhibitory activity also has allosteric nicotinic receptor modulation which could be particularly relevant in autism in view of nicotinic receptor pathology. It is effective as a cognitive enhancer in rodents (Table 4). As with donepezil, there are few studies of galantamine and autistic patients (Table 3). A placebo-controlled, double–blind, crossover, randomized, controlled trial with galantamine was conducted by Niederhofer et al. in 2002. Twenty boys (mean age = 7.4 years) with autistic spectrum disorder, diagnosed by ICD-10 criteria, were treated with placebo or galantamine. The ABC was used by parents and teachers to assess them. On average, the subjects receiving the placebo scored slightly higher than the subjects receiving galantamine. Improvements were seen in hyperactivity, inadequate eye contact and inappropriate speech. Clinicians' scores were not significantly different between the placebo and the galantamine groups. No side effects were reported, but most subjects had very limited language capacities. The study concluded that galantamine may be moderately effective in the short-term treatment of irritability in autism.

Galantamine has also been used to improve verbal skills in three autistic adults (Hertzman, 2003). Each patient exhibited an improvement in verbalization, and in some patients social behaviour improved. Only one person experienced side effects. An open-label trial by Nicolson et al. (2006) evaluated the use of galantamine with 13 children (mean age = 8.8 years) with autism. During the 12-week trial, parents scored their children monthly using the ABC and Conner's Parent Rating Scale – Revised. The children were also assessed by a clinician using the Children's Psychiatric Rating Scale and Clinical Global Impressions Scale. Eight of the 13 children (61.5%) were rated as responders to the drug. Improvements were seen with respect to irritability, social withdrawal, emotional liability, inattention and aggression on the various scales. There were no side effects reported except headaches in one patient.

Rivastigmine: Rivastigmine has been shown to improve impaired cognitive function in both rodents and subjects with dementia (table 5). Only one open-label study involving rivastigmine and autistic patients has been published (Chez et al., 2004; Table 3). About 32 autistic children (unspecified ages) were recruited to take part in a 12-week open-label trial. Patients were assessed using the Childhood Autistic Rating Scale, Gardner's Expressive and Receptive One-Word Picture Vocabulary tests, and the Conners Parent Rating Scale. Subjects were tested using these criteria at baseline and 6 and 12 weeks. Improvements were seen overall with regards to autistic behaviour, particularly in verbalization.

Combined use of ChEI with neuroleptic and antidepressant medication: Although the successes of most pharmacological treatments for autistic adults are limited, combining medications may prove to be more effective. A study by Wang et al., 2007 examined the synergistic effect of risperidone and galantamine on the phencyclidine-induced mouse model of schizophrenia. Similar to autism, schizophrenic patients suffer from cognitive defects and impaired memory function.

Table 4 Galantamine animal studies

Study	Design/procedure/variable	Dependent variable	Main findings
Barnes et al. (2000)	Thirty-two rats, randomized into three groups: control, galantamine (drug pumps) and donepezil. Pre-training around an 8-arm maze with 30-sec delay	Performance on 8-arm maze, nicotinic receptor density and affinity, spatial working memory (errors), and long-term potentiation decay times	Galantamine had no effect on performance levels on well-learned spatial memory task. Treatment with galantamine resulted in increased nicotinic receptors and extended long-term potentiation decay times
Van Dam et al. (2005)	Sixty rats, APP23 and wild type. Random assignment to galantamine (1.25 or 2.5 mg/kg). (Other AChE also tested)	Performance on the Morris water maze	1.25 mg/kg of galantamine-reduced cognitive deficits
Csernansky et al. (2005)	Mice were administered MK-801 (0.05–0.1 mg/kg) 30 min after galantamine (0.25, 0.50 or 1.00 mg/kg)	Behavioural tests to assess spatial learning, locomotion, fear conditioning, and shock sensitivity	Galantamine did not ameliorate MK-801-induced deficits in spatial reversal learning and in contextual and cued memory in dose-dependent manner. Similarly, it did not reverse MK-801-induced hyperlocomotion. However, galantamine altered shock sensitivity
De Bruin et al. (2006)	One-hundred seventy-two mice were used, randomized into four groups: control, effects of scopolamine, reversal of scopolamine by galantamine (0.63, 2.5 and 10 mg/kg) and effects of galantamine (0.63 mg/kg). Object recognition evaluated at retention intervals	Object recognition task	Galantamine partially reversed the effects of scopolamine, increasing performance to control levels. Mice administered with galantamine attained higher scores than controls

Table 4 (continued)

Study	Design/procedure/variable	Dependent variable	Main findings
Hernandez et al. (2006)	Three hundred and forty-four young (3–4 months) and aged (22–24 months) rats were randomly split, with subjects injected with saline (control) or drugs (galantamine – 1.5 or 3.0 mg/kg) twice daily for 15 days	Water maze testing, locomotor activity, and light/dark preference test	Rats treated with galantamine showed enhanced spatial learning
Dimitrova et al. (2006)	Rats randomly split into different groups with different conditions ($n = 8$) – i.e. hypoxia-induced rats + 0.5 mg/kg galantamine and hypoxia-induced rats + 1.0 mg/kg galantamine. Animals trained in shuttle box active avoidance test, step through and step down passive avoidance tests	Memory retention tests	In the step down passive avoidance test, rats treated with galantamine showed improvement during the learning and short-term/long-memory tests
Hohnadel et al. (2007)	Fifty rats administered with 0.3 mg/kg scopolamine, 0.5 mg/kg apomorphine or 0.1 mg/kg MK801 10 min prior treatment with 1 mg/kg galantamine	Prepulse inhibition (PPI) test.	Galantamine-improved PPI deficits in all three PPI disruption models

APP23, rat model of Alzheimer's disease showing disturbed behaviour.

Table 5 Rivastigmine animal studies

Study	Design/Procedure/Variable	Dependent variable	Main findings
Chen et al. (1998)	Unknown number of mice were administered with rivastigmine (1 or 2 mg/kg) 5 min after inducing closed-head injuries, tested daily for spatial memory	Morris water maze	Mice administered with rivastigmine (1 mg/kg) regained their performance levels 3 days after injury, control mice after 12
Bejar et al. (1999)	Unknown number of rats randomized into different groups. Administered with saline (control), rivastigmine (0.5–2.5 mg/kg) or tacrine, followed 10 min later by scopolamine. Spatial memory was tested 20 min afterwards	Morris water maze, passive avoidance tests	Rivastigmine antagonized the deficits induced by scopolamine
Wang et al. (2000)	Groups of 10 male or female rats administered with rivastigmine (0.75 mg/kg) or saline, administered 10 min later saline or scopolamine. The rats were subjected to the Morris water maze 20 minutes later	Performance on the Morris water maze	Rivastigmine was effective in antagonizing scopolamine-induced spatial memory impairment (more effective in the female rats)
Van Dam et al. (2005)	Sixty rats, APP23 and wild-type. Random assignment to rivastigmine (0.5 or 1.0 mg/kg). (Other AChE also tested)	Performance on the Morris water maze	0.5 mg/kg rivastigmine reduced cognitive defects

APP23, rat model of Alzheimer's disease with disturbed behaviour.

The administration of low doses of risperidone and galantamine (0.05 mg/kg) reversed the cognitive impairments induced in the mice by the phencyclidine. Treating the mice with SCH 23390, a dopamine D_1 receptor antagonist, and mecamylamine, a nicotinic acetylcholine receptor (AChR) antagonist, prevented the synergistic effect (scopolamine, a muscarinic AChR antagonist, had no effect after synergistic treatment). The successful synergistic effect may arise from the activation of nicotinic AChRs, which increase the activity of dopamine D_1 receptor neurotransmission (Wang et al., 2007). This study is a good example of how combining therapies may be beneficial to patients with cognitive disorders. Furthermore, this approach can be easily extended to concomitant use of ChEI and SSRIs, similarly to what has been recently used in elderly with Alzheimer's disease, which had

improvement in both cognitive functioning and functioning of daily living (Mowla et al., 2007).

Nicotinic Agonists

The activation of nicotinic acetylcholine receptors (nAChRs) activates the prefrontal cortex and enhances attention and cognition. Nicotine stimulates the release of several neurotransmitters in various brain areas, and the nAChRs stimulation by nicotine or the endogenous agonist, acetylcholine, induces a significant increase of glutamate in the layer V of the frontal cortex, via the involvement of α4β2 nAChRs (Lambe et al., 2003), receptors that are significantly depleted in autism. In clinical studies, nicotine has been documented to ameliorate some of the autistic spectrum symptomatology. Nicotine agonists (Gaynor and Handley, 2001) and nicotine patches improved attention and reduced complex tics in autistic Tourette's subjects (Howson et al., 2004). Interestingly, even the use of a single nicotine patch produced a significant reduction of the symptomatology with an average duration of 1–2 weeks post-application (Shytle et al., 1998). This effect is not mediated via the tryptophan metabolism and/or its catabolites (Gaynor and Handley, 2001).

Digoxin can also upregulate nicotine. Autistic children have higher levels of serum digoxin than controls, and nicotine is detected only in the plasma of autistic subjects (Kurup and Kurup, 2003). This study was conducted on young autistic and control subjects (age 10–15 years), all non-smokers. The authors of this study offered an explanation that the plasma nicotine detected only in the autistic children is due to the increase in tryptophan (a precursor of nicotine) and its catabolites and the reduction in tyrosine and its catabolites in patients' serum.

Nicotinic Cholinergic Antagonists: Treatment with nicotinic cholinergic antagonists may also alleviate autistic symptoms (Lippiello, 2005). The loss of nicotinic receptors from the frontal cortex, cerebellar cortex, and thalamus (summarized in Table 1) in autistic adults may have been a compensatory measure (or the result of negative feedback) for an excess of cholinergic neurons found in the enlarged young autistics' basal forebrain (Baumann and Kemper, 1994). Treatment with a nicotinic antagonist could potentially reduce the effects of the excess cholinergic neurons, preventing the reduction of nicotinic receptors in other parts of the brain.

As a nicotinic AChR agonist, nicotine stimulates dopamine release in the brain (Wonnacott et al., 2005). Dopamine agonists (reviewed in Möller et al., 2008) appear to exacerbate autistic symptoms, so a nicotinic antagonist would theoretically have therapeutic benefits. Mecamylamine is a nicotinic AChR agonist which could potentially be administered to autistics. No clinical trials with autistic patients have been conducted, but evidence from rodent studies offer support for a human trial. Recently, low doses of mecamylamine (0.125 mg/kg) administered to rats significantly improved cognitive function compared to saline-injected controls ($p < 0.05$; Levin and Caldwell, 2006).

Conclusions

While it is accepted that the cause of autism is heterogeneous, we are still far from understanding the specific causes of the condition and subsequently developing appropriate treatment. In autism, neuronal migrational arrest and changes in synaptic and dendritic arborization (Mukaetova-Ladinska et al., 2004) are accompanied by complex relationships between various interactive neurotransmitter systems. Since aetiological factors in autism remain unknown, treatment of the disturbing behavioural profile still largely remains symptomatic. The cholinergic deficit in autism and promising results of cholinergic drug trials so far indicate that further testing of ChEI to regulate the cognitive and associated behavioural changes in autistic subjects is needed. The use of ChEI (donepezil, galantamine and rivastigmine) in open-label trials is reported to improve core symptoms, but double-blind placebo trials are needed to provide more accurate information. A recent animal study using a pharmacologically induced deficit in prepulse inhibition suggested that cholinesterase inhibitors may have distinct profiles in regulating auditory sensory gating (Hohnadel et al., 2007), warranting further studies on similar deficits in the autistic spectrum disorder. The cholinergic abnormalities found in autistic patients' post-mortem, combined with the (partial) success of ChEI, may indicate a role for acetylcholine in the aetiopathology of autism. Although the case report studies have been largely conducted on adult autistic subjects, novel drug treatments utilizing the current knowledge may prove to be applicable for young autistic children, targeting core disease mechanisms.

Another therapeutic approach that can be implemented very early on is modifying the neurotransmission via change in diet. Thus, regulating the intake of tryptophan, tyrosine, and choline can be useful to regulate, for example, sleep and mood (Zeisl, 1986). Similarly, use of cholinomimetics may find a place in the treatment of some of the symptoms of the autistic spectrum disorders. Thus, a Russian study (Krasnoperova et al., 2004) explored the use of choline alfoscerate (CA; 400 mg/day) in 20 children (aged 3–8 years) with mild to moderate severity of autism. The treatment lasted 8 weeks, alongside with maintenance therapy with neuroleptics. Positive therapeutic effects were observed in 89% of treated autistic subjects: 61% had significant improvement, whereas minimal efficacy was observed in 28%. The clinical benefits of this improvement were: general improvement of behaviour, development of social and communicative skills, reduction of marked speech disturbances and enhancement of learning activity and productivity. The findings of this study suggest that CA is an effective and safe medicine for treatment of cognitive and behavioural disturbances in autism and can be used safely combined with additional neuroleptic therapy.

A parallel approach to cholinergic therapies is the use of cholinergic precursor loading strategy, involving choline and lecithin (Amenta and Tayebati, 2008). There is one open-label trial of choline in children with autism with positive outcome, although how this can be reconciled with the imaging data on elevated choline (above) is unclear. What we now need is controlled trials of diet in the autistic spectrum diseases, fully supported with biochemistry, to establish the role of change in

diet on behavioural and neurotransmitter changes in autism. In addition to possible dietary modulation of the cholinergic system there are also herbal medicines with relevant bioactivities. For example, numerous plants used in medicine have cholinesterase inhibitory activity (Houghton et al., 2006). Some of these such as sage (*Salvia officinalis*) and lemon balm (*Melissa officinalis*) have been shown to improve cognition and mood in normal adults (Kennedy et al., 2003, Tildesley et al., 2003, 2005; Scholey et al., 2008). Since they are without adverse side effects at standard doses, such agents may also be worth considering in autism spectrum disorders.

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Oxytocin and Autism

Peter Kirsch and Andreas Meyer-Lindenberg

Introduction

Impairments in social interaction have been regarded as a central feature of autism from the initial description by Kanner to the currently used diagnostic definition. Therefore, it made and still makes sense to study the neurobiology of social attachment to define targets potentially relevant to the pathogenesis and treatment of this still enigmatic group of disorders. Since the prosocial neuropeptide oxytocin (OT) is central to social processes throughout the animal kingdom, several authors (Modahl et al., 1992; Panksepp, 1993; Insel et al., 1999; Hammock and Young, 2006; Carter, 2007) have speculated about a role for OT in autism, starting with a letter to the editors of the *Journal of Autism and Developmental Disorders* by Charlotte Modahl and colleagues. In this chapter, we will first review the evidence for the neurobiological function of OT in man vis-à-vis the general neuroscientific interest in neural substrates of social behavior, focusing on the cognitive subprocesses for social information processing and their neural correlates (Adolphs, 2003; Insel and Fernald, 2004). This is followed by the evidence for a relevance of OT for autism pathogenesis and therapy.

Neurobiology of Oxytocin: Preclinical Studies

OT is a nine amino acid neuropeptide that, together with the other socially relevant neuropeptide vasopressin, is synthesized in the neurons of the paraventricular and supraoptic nuclei of the hypothalamus. After its production, oxytocin is transported along the axons of these neurons, stored in their vesicles, and finally segregated by the posterior pituitary gland. Peripherally, oxytocin stimulates the contraction of the uterus during labor and also the contraction of

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the mammary glands increasing the ejection of milk during suckling. The oxytocin plasma level is increased during suckling (McNeilly et al., 1983) and both the male and the female orgasm (Carmichael et al., 1994; Blaicher et al., 1999). In general, the hormonal as well as the neurotransmitter effects of oxytocin can be summarized as related to reproduction (Gimpl and Fahrenholz, 2001).

The effects of oxytocin in the central nervous system are mediated by the oxytocin receptor (encoded by *OXTR*) in the brain (Buijs et al., 1985; Gimpl and Fahrenholz, 2001). In most species, OT receptors can be localized in the hypothalamus, the thalamus, the hippocampus, the mesencephalon, the brain stem, and especially in the amygdala. However, there seems to be considerable species differences in *OXTR* expression. In humans, the database on the regional expression is unfortunately scarce. The only postmortem study in humans published by Loup and colleagues (Loup et al., 1991) found specific binding sites of OT in several nuclei of the forebrain, especially the nucleus of Meynert, the vertical nucleus of the diagonal band, and the ventral part of the lateral septal nucleus, as well as in the hypothalamus, the globus pallidus, and the brain stem but not in the amygdala. Because their results are in contradiction to studies in other species, a replication of their findings seems to be urgently needed, especially with respect to results of the in vivo studies reported below.

Especially using rodent models, including prairie voles showing considerable pair and maternal bonding, OT has now been shown to be relevant for the modulation of complex emotional and social behaviors such as maternal behavior (Pedersen and Prange, 1979), attachment (Insel and Young, 2001), social exploration, recognition (Winslow and Insel, 2004), trust (Kosfeld et al., 2005), and aggression (Liebsch et al., 1996; McCarthy et al., 1996; Appenrodt et al., 1998; Bosch et al., 2005), as well as anxiety (Liebsch et al., 1996; McCarthy et al., 1996; Appenrodt et al., 1998), fear conditioning (Stoehr et al., 1992), extinction (Ibragimov, 1990), and reduced stress responses and fear (McCarthy et al., 1996; Windle et al., 1997).

Animal models strongly suggest that the central role of oxytocin in mediating complex social behavior depends on the function of the amygdala: oxytocin acts on the amygdala to reduce fear (McCarthy et al., 1996; Windle et al., 1997) and modulate aggression (Bosch et al., 2005), and knockouts for the oxytocin receptor in mice show a profound social recognition deficit despite normal olfactory and spatial learning abilities that can be fully restored by injection of oxytocin in the medial amygdala (Ferguson et al., 2001). Huber and colleagues (Huber et al., 2005) described the potential neurophysiological mechanism of the oxytocin effect in the amygdala. They not only replicated the finding that oxytocin receptors are highly expressed in the rat amygdala but also demonstrated that oxytocin acts on the central amygdala to inhibit excitatory flow from the amygdala to brain stem sites mediating fear response. Together, this provides an attractive regional a priori hypothesis for the action of OT in human brain, although OT binding sides were not identified in the human amygdala in the one study available on the subject (Loup et al., 1991).

Neurobiology of Oxytocin Action in Humans

While the study of the effects of these neuropeptides in animals is comparatively straightforward, human experiments have been hampered by the fact that OT, being a peptide, is unlikely to pass the blood–brain barrier and cannot be ingested. A new era of OT studies was enabled when it was shown that neuropeptides like OT enter the brain when given intranasally (Born et al., 2002) by a mechanism that remains still unclear (but might involve either an incomplete blood–brain barrier in the olfactory region or a large increase of OT in the venous plexuses at the base of the skull pronounced enough to overcome the barrier). Using this technique in humans, it could be demonstrated that oxytocin reduces cortisol release during a stress task (Heinrichs et al., 2003), has anxiolytic effects (McCarthy et al., 1996; Heinrichs et al., 2003; Domes et al., 2007a) and, in a landmark study, increases interpersonal trust in a social exchange game (Kosfeld et al., 2005). These patterns of effects strongly suggest a central role of the amygdala in the oxytocin effects on human social behavior, since amygdala activation is linked to trust (Winston et al., 2002) – presumably because of its role in danger monitoring. To test the central role of the amygdala for the psychological effects of oxytocin in humans in vivo, we studied amygdala circuitry after double-blind crossover intranasal application of placebo or oxytocin (Kirsch et al., 2005).

We employed two visually matching tasks requiring visual processing of threatening stimuli of different social valence that reliably engage the amygdala and have been shown to be sensitive to genetic mechanisms of abnormal social behavior in patients with Williams-Beuren syndrome (Meyer-Lindenberg et al., 2005) and persons with an increased risk for anxiety (Pezawas et al., 2005). In one experimental condition, one of two simultaneously presented angry or afraid faces was matched with an identical target face (Hariri et al., 2002). In the other experimental condition, participants matched one of two simultaneously presented fearful/threatening scenes from the International Affective Picture System (IAPS) (Lang et al., 1997) with an identical target scene (Hariri et al., 2003). As a control condition, participants matched simple shapes (circles or ellipses). The scenes were specifically selected to be devoid of social interaction or facial displays and should therefore represent socially irrelevant threatening stimuli, while threatening and angry faces are socially relevant stimuli, an assumption supported by previous research with this paradigm (Meyer-Lindenberg et al., 2005). In a double-blind crossover experiment, 15 participants applied oxytocin or placebo intranasally, a modality shown to reliably deliver neuropeptides to the brain (Born et al., 2002). In behavioral testing, the neuropeptide had no effect on task performance, anxiety scales, or arousal, in agreement with previous reports (Kosfeld et al., 2005). In the fMRI experiment, strong, right-lateralized activation of the amygdala to both classes of stimuli was observed during the placebo condition (Fig. 1), confirming previous results (Hariri et al., 2002). Compared to placebo, oxytocin significantly depressed amygdala activation. Testing the stimulus types separately showed that this effect was more pronounced for faces (socially relevant stimuli) than scenes (Fig. 1C). However, this did not represent an interaction as the slope of the effect was almost identical for the two stimulus classes

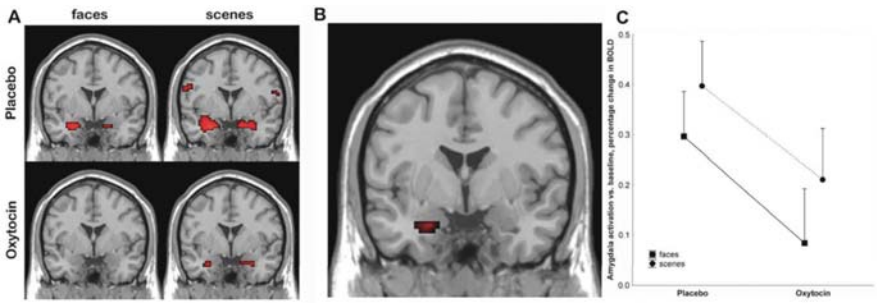

Fig. 1 Oxytocin effects on amygdala activation. (A) Rendering on normal coronal MRI at the level of the anterior commissure (in neurological orientation: brain left is on viewer's left). Response to face stimuli left, scene stimuli right. *Top*: placebo, *bottom*: oxytocin. See Table 2 for statistical information. (B) Significantly higher activation under placebo than oxytocin (main effect of drug condition). (C) Plot of *bold* in amygdala region of interest. From Kirsch et al. 2005, with permission

(Fig. 1C). Our finding of reduced amygdala activation under oxytocin confirmed our hypothesis about the central role of the amygdala for the psychological effects of oxytocin like reduced fear and increased trust. In addition, reduced amygdala activation to fearful faces has been linked to increased sociability and decreased social fear in humans (Adolphs et al., 2005; Meyer-Lindenberg et al., 2005), and the neuropeptide action observed here may therefore contribute to the prosocial effects of oxytocin. It is intriguing to note that the reduction of amygdala activation was more pronounced for socially relevant stimuli (faces) than for the socially less relevant scenes; while this effect did not reach statistical significance and did not represent an interaction effect, differential impairment of amygdala signaling related to the social relevance of the stimuli is in agreement with emerging primate lesions (Prather et al., 2001) and human (Meyer-Lindenberg et al., 2005) data indicating that social and nonsocial fear may depend on dissociable neural systems.

These initial findings have now been replicated and extended. Petrovic and colleagues (Petrovic et al., 2008) also found a reduction of amygdala activation to faces presented during an evaluative conditioning paradigm. The reduction was accompanied by an attenuation of negative ratings of these aversively conditioned faces. While these authors stress the prosocial effect of oxytocin, Domes and coworkers (Domes et al., 2007b) found a more general effect of oxytocin on the processing of socially relevant stimuli. They also replicated the attenuating effect of oxytocin on the amygdala activation during the presentation of emotional faces. However, in contrast to our study (Kirsch et al., 2005) they presented faces of different valence and found reduced amygdala activation for all conditions. This result is in concordance with the interpretation that the amygdala is responding to any potentially relevant social and emotional stimuli (Davis and Whalen, 2001). Finally, in a recent study by the Fehr group (Baumgartner et al., 2008), a social exchange paradigm was used during fMRI to show that difference in trust adaptation was

associated with a specific reduction in activation in the amygdala, but also the midbrain regions and the dorsal striatum. All findings therefore support the assumption that the reduction of amygdala signaling under OT challenge is facilitating social approach behavior but also point toward the relevance of a distributed neural network of which amygdala is part, as expected from the complexity of the set of behaviors that this hormone can modulate. This becomes most clear in the recent study (Domes et al., 2007b), which demonstrated improved performance in a theory of mind task, the Reading the Mind in the Eyes Test, after a single dose of oxytocin, since mind reading is a task that has been repeatedly associated with amygdala activation (Baron-Cohen et al., 1999; Stone et al., 2003). Therefore, at least in controls, the observed effect of oxytocin on mind reading should not be mediated by amygdala attenuation. However, conclusions about the neurobiological basis of the oxytocin effect on mind reading can only be drawn from neuroimaging studies directly investigating the changes of brain activation during challenge.

The relevance of OT for human social behavior is further supported by recent evidence that variation in the gene for the OT receptor, *OXTR*, is associated with social phenotypes. Prichard et al. (Prichard et al., 2007) found that a polymorphism in *OXTR* was associated with earlier age of having children in women, while another group demonstrated an impact of another variant (interestingly, a SNP also associated with autism; see below) in *OXTR* with maternal sensitivity (Bakermans-Kranenburg and van Ijzendoorn, 2008).

Oxytocin and Autism: Preliminary Evidence and Neurobiological Mechanisms

While human studies are therefore homing in on neural circuits for social behavior impacted by OT, what is the evidence for an involvement of this hormone in autism and ASD? Several lines of clinical and preclinical evidence support this hypothesis. First, plasma oxytocin levels were found to be lowered in autism and correlated with social impairment (Modahl et al., 1998; Green et al., 2001). These findings are interesting; however, it raises the question as to how well plasma OT correlates with brain levels of OT, an open question in humans. Second, the gene for the oxytocin receptor, *OXTR*, is located at a suggestive linkage peak for the disorder (McCauley et al., 2005). Third, a number of clinical association studies suggest a relation between a genetic variation in *OXTR* and autism (Wu et al., 2005; Jacob et al., 2007; Lerer et al., 2007; Yrigollen et al., 2008). Finally, an intriguing case report of duplication in the 3p25 region, associated with a twofold increase of *OXTR* expression relative to controls and an autism-like phenotype, has appeared (Bittel et al., 2006).

Clearly, these are preliminary findings that need to be confirmed, extended by genome-wide association study, and examined with regard to the neurophysiological underpinnings. For the latter approach, the importance of OT-related signaling

in the amygdala for social processing might in fact provide a neurophysiological link between oxytocin deficit and social impairment in autism. It is known that the amygdala shows an abnormal development in autism with an enlargement during childhood (Sparks et al., 2002; Schumann et al., 2004) which seems to be associated with increased anxiety (Juranek et al., 2006) and impaired social and communication abilities (Munson et al., 2006). Interestingly, the relation between volume deviations and anxiety or social impairments seems to be stronger for the right amygdala, the site which also showed a stronger response to oxytocin in our fMRI study (Kirsch et al., 2005). In contrast, during adulthood a reduced amygdala volume was observed in autism (Aylward et al., 1999) which might contribute to impaired social functioning (Nacewicz et al., 2006). In general, the anatomy of the amygdala seems to be changed in autism (for a comprehensive overview, see Amaral et al., 2008) which is also supported from postmortem data revealing microscopic pathology in the amygdala in autism (Rapin and Katzman, 1998). However, evidence for an important role of the amygdala in autism comes not only from anatomical data but also from functional studies (Baron-Cohen et al., 2000). Several social functions like face recognition and mental state consideration (mind reading, theory of mind) that are impaired in autism are known to be related to amygdala activation. Wang and coworkers (Wang et al., 2004) found no modulation of the amygdala during a facial emotion recognition task in children with an autistic spectrum disorder but not in control children. Critchley and colleagues (Critchley et al., 2000) found a lack of amygdala activation during the processing of emotional face expressions in high-functioning adults with an autistic disorder. Baron-Cohen and colleagues (Baron-Cohen et al., 1999) found a reduced activation of the amygdala in high-functioning autism patients during a mind reading task. Interestingly, it was shown that individuals with autism misjudge the trustworthiness of human facial expressions (Adolphs et al., 2001) with a comparable performance to neurological patients with amygdala lesions. However, deficient amygdala activation in autism seems not to be a consequence of reduced amygdala activation in general. In other socially relevant tasks, not including the demand to infer about other persons state, a hyperactivation can be observed in autism. Dalton and colleagues (Dalton et al., 2005) found increased amygdala activation in autistic probands during gaze fixation. Since autistic persons usually try to avoid gaze fixation, the authors interpreted their finding as reflecting increased emotional responding. Different reasons may account for these contradictory findings. One reason might be the different task type or difficulty leading to a different involvement or strategy of the patients in the task. In all studies, patients showed a poorer performance than controls which might indicate that the patients were prevented from using an amygdala-based strategy but used alternative networks to solve the task. However, when confronted with an explicit social stimulation like gaze fixation, hyperactivation might occur. Another explanation might be the fact that autism is a very heterogeneous disorder. It could be speculated that a specific subgroup of patients with a distinct social fear shows amygdala hyperactivation. These patients might show the strongest impairment of their oxytocin system and might therefore especially benefit from an oxytocin treatment.

Second, imaging genetics can also be used to study the effects in brain of the genetic variation in *OXTR* linked to autism in clinical studies. A model for these studies that also provides independent evidence for an association between amygdala, prosocial neuropeptides, and autism comes also from our recent functional genetics study (Meyer-Lindenberg et al., 2008) of the sister hormone of oxytocin, vasopressin. Genetic variations in the gene for the brain receptor of vasopressin, *AVPR1A*, have in fact been implicated in autism (Kim et al., 2002; Wassink et al., 2004; Yirmiya et al., 2006). As with oxytocin, this evidence is further enhanced by linkage to the region of the gene (Wassink et al., 2004) and social dysfunction reminiscent of autism found in *AVPR1A* knockout mice (Bielsky et al., 2004). In our study we found that two microsatellite polymorphisms of the vasopressin receptor gene (*AVPR1A*), RS1 and RS3, encoding the receptor subtype most heavily implicated in behavior regulation and previously linked to autism and behavioral traits, predicted amygdala activation. We used an imaging genetics approach in a sample of 121 volunteers and presented the emotional face-matching paradigm described above. We found differing activation of the amygdala in carriers of risk alleles for RS3 and RS1. Carriers of the 334 bp risk allele of RS3 showed significantly increased left amygdala activation and carriers of the 320p allele of RS1, which is undertransmitted in autism, showed a reduced activation. Furthermore, we identified a functional difference in human brain between short and long repeat lengths. While for RS3 the long variant was associated with increased amygdala activation for RS1 the shorter variants showed hyperactivation of the amydala. This mirrors findings in prairie voles (Hammock and Young, 2005) and supports these authors' hypothesis that length variation in this locus is related to sociability. Studies applying this approach to *OXTR* variants, previously discussed, that are associated with autism, are currently ongoing.

Given these relations for oxytocin and its suitability to modulate the amygdala and autism, it is the next logical step to think about an oxytocin treatment in autism. Specifically, the strand of research reviewed above on amygdala attenuation under oxytocin would suggest that dampening of this structure would be beneficial and increase trust, provided it is assumed, based on the findings of Dalton et al. discussed above, that at least a subgroup of patients with autism have hyperreactivity in this structure. Also, the finding of Domes and colleagues (2007a) who were able to improve the performance in a mind reading task, the "Reading the Mind in the Eyes" test, after a single dose of oxytocin, would point to a beneficial effect of OT treatment, even though the mechanism for improved theory of mind by OT remains unclear.

Studies directly investigating the effect on oxytocin autistic symptoms were pioneered by Eric Hollander and colleagues. In 2003 they reported a reduction of repetitive behaviors in 15 adults with autism after an oxytocin infusion (Hollander et al., 2003). Using a comparable mode of application, they could recently also demonstrate an effect of oxytocin treatment on social cognition in terms of affective speech comprehension (Hollander et al., 2007). Interestingly in this study, those patients who received oxytocin first showed a prolonged effect of the treatment while those patients who received placebo first showed a fast reversal of their ability

to correctly assign emotional meaning to speech intonation, pointing to a possible effect of OT on neural plasticity in systems for social cognition in autism. So far, it is not fully understood how peripherally administered oxytocin can impact the central nervous system, since it could not be shown that is passes the blood–brain barrier. Therefore, the intranasal application of neuropeptides seems to be a more promising approach, and studies using this mode of administration are ongoing. However, since half-life of intranasal oxytocin is very short, new developments, such as longer acting or non-peptide OT receptor agonists that can be applied orally or systemically, as well as placebo-controlled studies are needed to confirm the positive effect of oxytocin on autism symptoms. Clearly, given that autism is a biologically heterogeneous phenotype, a single or unique treatment of the disorder cannot be expected, especially due to its neurodevelopmental component. Nevertheless, oxytocin as a treatment might be one promising component of a treatment program. If it turns out that not all patients benefit from such a treatment, effective patient stratification procedures will be needed to identify those patients that might profit. The reviewed brain imaging work showing differential responses of the amygdala, and the limbic circuits in which it participates, under placebo or oxytocin challenge might be a promising way to identify those (for example, by selecting patients showing hyperresponsivity of amygdala to social threat stimuli). Furthermore, this approach can be combined with the identification of genetic variants whose carriers have the greatest likelihood of benefiting from oxytocin challenge. While there is still a long way to go to translate the intriguing findings on the relation between oxytocin, social cognition, and the amygdala to an effective treatment for autism, prosocial neuropeptides represent one of our best leads for evidence-based therapy of this severe and disabling disorder.

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The Role of the Noradrenergic System in Autism Spectrum Disorders

David Q. Beversdorf

Autism is characterized by impairments in socialization, communication, and the presence of stereotyped and repetitive behaviors with onset before 3 years of age (American Psychological Association, 1995). Asperger syndrome is characterized by preserved language in the presence of the other characteristics of autism (American Psychological Association, 1995). However, in patients who initially meet the criteria for autism, the symptoms may evolve such that they no longer meet all criteria for autism as they develop from childhood to adulthood (Seltzer et al., 2003). Therefore, the term autism spectrum disorder (ASD) is often utilized to collectively describe both syndromes (Beversdorf et al., 1998).

Noradrenergic System and Autism

Agents that decrease the activity of the noradrenergic system are widely used for anxiolytic and behavioral purposes in ASD. Benefits in language and social behaviors have been reported in a consecutive case series with beta-adrenergic antagonists (Ratey et al., 1987). Benefits have also been reported with other anxiolytics that act on the noradrenergic system, such as the alpha-2 adrenergic agonists, which act to presynaptically inhibit norepinephrine release, where improvements in hyperactivity, impulsivity, hyperarousal, and social relationships were observed in double-blinded placebo-controlled crossover trials (Jaselskis et al., 1992; Fankhauser et al., 1992), and with marked improvement in behavior and verbal response in one reported case (Koshes and Rock, 1994). A number of researchers have demonstrated findings suggestive of increased noradrenergic activity in autism, including increased plasma epinephrine and norepinephrine (Lake et al., 1977; Launay et al., 1987), and altered urinary excretion of various catecholaminergic metabolites (Barthelemy et al., 1988; Martineau et al., 1992). However, there

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may be a number of alternative explanations for these findings. Subsequent studies demonstrating no abnormalities in basal noradrenergic functioning have led to the suggestion that increased reactivity to clinical procedures such as blood drawing and urine collection in autism may have led to the earlier atypical findings (Minderaa et al., 1994). Furthermore, pathology is not found in the volume, cell counts, or cell density in postmortem tissue from the locus coeruleus in autism (Martchek et al., 2006). Others, though, have proposed that the behavioral effects of fever in autism (Curran et al., 2007) may be related to normalization of a developmentally dysregulated noradrenergic system in autism (Mehler and Purpura, 2009). However, regardless of the ambient activity of the noradrenergic system in autism, other evidence suggests a potential benefit from noradrenergic blockade in ASD.

Noradrenergic System, Stress, and Cognition

Research involving adolescents with stress-induced cognitive impairment without neurodevelopmental diagnoses demonstrated that treatment with the beta-adrenergic antagonist propranolol significantly improved scores on the Scholastic Aptitude Test (SAT) (Faigel, 1991). This finding suggests a role of the noradrenergic system in stress-related modulation of performance in some types of problem solving in certain individuals. Propranolol has also demonstrated efficacy in stress-induced impairment in performance on other tasks including public speaking in anxiety-prone individuals (Lader, 1988; Laverdue and Boulenger, 1991).

The anagram task has been widely used in studies of anxiety, demonstrating a decrement in performance in anxious subjects (Harleston et al., 1965; Tomasini, 1973; Dey, 1978), and has furthermore been proposed as a marker of anxiety (Thyer and Papsdorf, 1982), suggesting its utility in research on the influence of stress. An increase in activity of the noradrenergic system is known to occur in the setting of stress (Ward et al., 1983; Kvetnansky et al., 1998), and situational stressors have been shown to impair performance on other tests of cognitive flexibility (Martindale and Greenough, 1975). Therefore, the anagram task was utilized in studies investigating the effects of noradrenergic agents on network flexibility in verbal problem solving. Anagram performance is significantly better after administration of beta-adrenergic antagonists (propranolol) than after noradrenergic agonists (ephedrine) (Beversdorf et al., 1999). Follow-up studies have demonstrated that noradrenergic modulation of cognitive flexibility appears to be mediated by a central mechanism rather than a peripheral mechanism, since performance is significantly better after propranolol (central and peripheral beta-adrenergic antagonist) than after nadolol (peripheral-only beta-adrenergic antagonist) (Beversdorf et al., 2002). A central-only mechanism would be predicted by the modulatory effect of norepinephrine on the signal-to-noise ratio of neuronal activity within the cortex (Hasselmo et al., 1997) and the correlation between electronic coupling of noradrenergic neurons in the monkey with proportions of goal-directed versus exploratory behaviors (Usher et al., 1999). However, in each of these studies involving anagrams, whereas performance on propranolol was significantly better than on ephedrine or nadolol,

it did not significantly differ from placebo (Beversdorf et al., 1999; Beversdorf et al., 2002). Subsequent research demonstrated that propranolol is beneficial for network flexibility in problem solving particularly when the subject is struggling with the problem (Campbell et al., 2008), as would be expected since greater flexibility would be required for such situations where a greater network search is needed, and can actually impair performance when subjects are solving problems with ease (Campbell et al., 2008). However, in patients where noradrenergic activity is upregulated, such as in cocaine withdrawal, propranolol benefits performance on the simplest problems (Kelley et al., 2007). Further research examined the interaction between propranolol and the cognitive effect of stress in individuals without any history of anxiety-related disorders, revealing impairment in anagram performance as well as other tasks involving semantic network flexibility under conditions of stress, which is reversed by propranolol (Alexander et al., 2007). Therefore, this pharmacology-stress interaction effect on cognition may represent a fundamental aspect of cognition in typical individuals and does not require the presence of an anxiety-related disorder or noradrenergic dysregulation.

Noradrenergic System, Cognition, and Autism

A range of theories have been proposed to account for the cognitive impairment in autism, including inability to comprehend the perspectives of others ("theory of mind") (Baron-Cohen et al., 1985), inability to utilize context in understanding the environment ("central coherence") (Frith and Happé, 1994; Happé, 1994), inability to process emotional information (Fotheringham, 1991; Hobson, 1991; Hobson, 1993; Beversdorf et al., 1998), impaired executive function (Rumsey, 1985; Rumsey and Hamburger, 1988; Rumsey and Hamburger, 1990), and global/local processing biases toward the local (Mottron and Burack, 2001), among others. As a manifestation of decreased utilization of context, research has supported the hypothesis that individuals with ASD have a restriction of flexibility of access to the semantic network, including decreased semantic clustering in verbal memory (Minshew and Goldstein, 2001), lack of increased recall of words when syntactic and semantic context is added (O'Connor and Hermelin, 1967; Hermelin and O'Connor, 1970; Hermelin and Frith, 1991), as well as superior performance on the "false memory" task (where the semantic and associative relationships between a heard word list and a not presented lure induce a false memory for the lure in typical individuals) (Beversdorf et al., 2000), despite a more typical performance by the same participants on most other cognitive tests for autism (Beversdorf et al., 1998). Superior performance in ASD has also been detected for "false memory" for a visuospatial task (Hillier et al., 2007). Recently, research using functional connectivity fMRI (fcMRI) has demonstrated a potential neural substrate for such decreased semantic network flexibility by showing that the interrelation between active brain regions during sentence comprehension is decreased in ASD (Just et al., 2004). Individuals with ASD do utilize semantic information to some extent in memory performance, as is demonstrated by previous research including other "false memory" studies

(Bowler et al., 2000), but not to the same degree as typical subjects. An agent that could affect the semantic network and other information networks might be of benefit in ASD. Therefore, we examined the effects of pharmacological agents on network flexibility among individuals with ASD. Specifically, we examined the effect of propranolol on network flexibility in high-functioning adults with ASD using simpler verbal problem-solving tasks with which individuals without neurodevelopmental diagnoses would not be expected to benefit (Campbell et al., 2008). Due to their decreased flexibility of access to networks, we expected that individuals with ASD would have a selective performance benefit from propranolol.

As expected based on our previous examination of the interaction between task difficulty and the effects of propranolol on network access in verbal problem solving (Campbell et al., 2008), our pilot study demonstrated an impairment in performance on these simple anagrams with propranolol in individuals without neurodevelopmental diagnoses. However, as indicated by a significant drug by group interaction, the effect of drug on performance in individuals with ASD was significantly different from controls, with ASD subjects performing better with propranolol on the same task (Beversdorf et al., 2008). This finding was present despite no significant difference between groups in overall performance on the anagrams in the placebo condition as well as no significant difference in age or IQ. Similar benefits were also observed on semantic fluency with propranolol in ASD in further pilot studies (Scaduto et al., 2008).

Previous work has demonstrated that whereas effects of propranolol on the networks in verbal problem solving can be difficult to detect in individuals without neurodevelopmental diagnoses (Beversdorf et al., 1999; Beversdorf et al., 2002; Campbell et al., 2008), a beneficial effect of the drug occurs in the setting of a psychosocial stressor (Alexander et al., 2007) and cocaine withdrawal (Kelley et al., 2007) believed to be due to noradrenergic upregulation, as well as individuals without such stressors when encountering more difficult problems (Campbell et al., 2008). Verbal problem solving is also affected by alterations in noradrenergic activity induced by changes in posture (Lipnicki and Byrne, 2005), sleep phase (Stickgold et al., 2001), and in vagal nerve stimulation (Ghacibeh at el., 2006). A beneficial effect from propranolol can also be detected in Broca's aphasia patients struggling to perform a naming task (Beversdorf et al., 2007b). Whereas it is not certain whether norepinephrine is upregulated in autism (Lake et al., 1977; Launay et al., 1987; Barthelemy et al., 1988; Martineau et al., 1992; Minderaa et al., 1994; Martchek et al., 2006) or whether the restriction is more anatomical in nature (Belmonte et al., 2004), our preliminary findings begin to suggest that propranolol also has some benefit for performance of the hyper-restrictive networks proposed by network models of autism (Cohen, 1994; McClelland et al., 2000; Beversdorf et al., 2007a) (see Fig. 1). In our studies, baseline heart rate and blood pressure were similar between groups, offering some suggestion against our finding resulting from a higher baseline stress level in the ASD population. However, this will warrant further investigation using other measures of stress response. Future work will also be needed in a larger sample of individuals to further determine response to propranolol on a range of tasks in ASD.

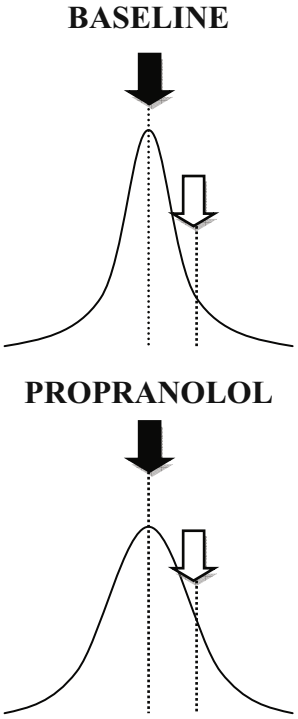

Fig. 1 Theoretical proposed representation of the signal to noise in the cortical networks as affected by propranolol, based upon the findings of Hasselmo et al (1997) on the effects of norepinephrine in the cortex. *Black arrows* indicate a greater response to the most dominant signal input, such as representation of an attended stimulus, which may be suppressed by noradrenergic blockade. *White arrows* indicate the response to nondominant signal input, such as intrinsic or associative fiber inputs, the "noise" in the model, which may increase with noradrenergic blockade, proposed to be how problems without an immediately accessible answer may be solved more readily in this condition or how patients with impaired flexibility of network access may benefit in a more general manner in this condition.

Mechanism of the Effects of the Noradrenergic System on Cognition

Norepinephrine is a critical component of the arousal mechanism (Smith and Nutt, 1996; Coull et al., 1997; Coull et al., 2004). The prefrontal cortex, believed to be important for various types of cognitive flexibility (Vikki et al., 1992; Karnath and Wallesch, 1992; Eslinger and Grattan, 1993; Duncan et al., 1995; Robbins, 2007), has afferent projections to the locus coeruleus in primates (Arnsten and Goldman-Rakic, 1984), which contains a majority of the noradrenergic neurons in the central nervous system and sends extensive efferents throughout the brain (Barnes and Pompeiano, 1991). A range of other cognitive effects have also been described with noradrenergic agents, including effects on motor learning (Foster et al., 2006),

response inhibition (Chamberlain et al., 2006a), working memory, and emotional memory (Chamberlain et al., 2006b).

Anagrams involve a search through a wide network in order to identify a solution ("unconstrained flexibility"), which appears to be modulated by the noradrenergic system, whereas other cognitive flexibility tasks such as the Wisconsin Cart Sort Test (Heaton, 1981) involve set shifting between a limited range of options ("constrained flexibility"), which may not be modulated by the noradrenergic system in the same manner, and may even benefit from increased noradrenergic activity (Usher et al., 1999; Aston-Jones and Cohen, 2005). Specifically, as described above, decreased noradrenergic activity appears to benefit tasks such as anagrams when subjects are struggling or challenged by stressors (Alexander et al., 2007, Campbell et al., 2008), whereas increased set switching on a two alternative forced choice task is associated with increased noradrenergic tone in primate studies (Usher et al., 1999; Aston-Jones and Cohen, 2005). "Constrained" flexibility can be further divided into intradimensional and extradimensional set shifting (Robbins, 2007). The dopaminergic system appears to affect intradimensional set shifting (Robbins, 2007), while the noradrenergic system, specifically by action on the alpha-1 receptor, appears to modulate performance on extradimensional set shifting (Lapiz and Morilak, 2006; Robbins, 2007). However, the beta adrenergic receptors in the noradrenergic system appear to modulate the "unconstrained" flexibility (Beversdorf et al., 1999; Beversdorf et al., 2002; Alexander et al., 2007).

Whereas the anatomical pathways by which the brain utilizes network flexibility in problem solving, such as in anagrams, is not yet understood, the frontal lobes appear to play a crucial role (Vikki et al., 1992; Karnath and Wallesch, 1992; Eslinger and Grattan, 1993; Duncan et al., 1995). In general, frontal brain regions, likely the dorsolateral prefrontal cortex, may guide the search by selective engagement of the posterior regions relevant to the type of problem being solved. EEG data is supportive of a strong frontal–posterior network as evidenced by the strong coherence of tracings of such regions during "creative" tasks in various modalities (Petsche, 1996). Further support for this comes from evidence that right frontal lesions impair strategy shifting ability in patients, whereas parietal lesions result in a general visuospatial information processing impairment in patients using a spatial task derived from an unconstrained visuospatial flexibility task, the Matchstick Test of Cognitive Flexibility (Guilford, 1967; Miller and Tippett, 1996). Therefore, propranolol might be expected to affect the interactions between these anterior and posterior brain regions.

Future Directions

As autism is characterized by decreased functional connectivity between such distant brain regions in both language and spatial problem-solving tasks (Just et al., 2004; Just et al., 2007), future functional neuroimaging studies using pharmacological modulation with noradrenergic agents may begin to address how agents such

as propranolol might affect cognition in ASD. Further research also will need to investigate the range of noradrenergic and anxiolytic agents that have this effect in ASD, the range of cognitive tasks affected in this manner, the relationship between these findings and stress responses in the testing environment in ASD, and the relationship between these results and the previously reported clinical benefits of noradrenergic agents in ASD (Ratey et al., 1987; Jaselskis et al., 1992; Fankhauser et al., 1992; Koshes and Rock, 1994), as well as the potential for benefit from such agents in lower functioning patients, and in a younger patient population while learning is still in its earlier stages. The effects of various challenges to the noradrenergic system and their effects will also be an important avenue for future research (Mehler and Purpura, 2009).

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Oxidative Stress in Autism and Its Implications for Dopamine-Stimulated Phospholipid Methylation

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Many biochemical reactions involve reduction or oxidation, the gain or loss of electrons, respectively. This is especially true in aerobic organisms, such as humans, who utilize oxygen as a primary source of energy, creating a constant source of oxidative risk that must be counterbalanced by an effective antioxidant redox buffering system (Benzie, 2000). Since the origin of life, molecules containing reduced sulfur (e.g., thiols) have served as primary antioxidants, based upon the ease with which they release their hydrogen atom, making them excellent reducing agents. Indeed, the simplest thiol, hydrogen sulfide, a gas released from underwater volcanic vents, may have been essential for earliest life forms (Clark et al., 1998). When thiols within proteins (e.g., enzymes) react with oxygen, it can exert important regulatory effects, allowing metabolism to be responsive to redox status.

Glutathione (GSH), a tripeptide containing the sulfur amino acid cysteine, along with glutamate and glycine, is the primary redox buffer in all human cells, and levels of cysteine are rate limiting for GSH synthesis (Fig. 1). When two molecules of GSH combine to form oxidized glutathione (GSSG), two reducing equivalents of H^+ are made available for quenching of reactive oxygen species (ROS), thereby protecting cells from oxidative damage. GSH also reacts with xenobiotics and heavy metals, increasing their rate of excretion and providing an important mode of detoxification.

Intracellular levels of GSH are very high (1–10 mM), and a considerable proportion of cellular metabolism is devoted toward maintaining adequate GSH levels (Akerboom et al., 1982). The ratio of GSH to GSSG is an index of cellular redox status, and any significant reduction in GSH/GSSG places the cell in a state of oxidative stress, which can ultimately lead to cell death. However, gradual increases in oxidative stress cause alterations in enzyme function, cellular signaling, and gene expression that serve to shift cellular activity toward antioxidant needs, combating oxidative stress, and restoring normal redox status. Although these countermeasures may be effective in bringing GSH/GSSH back to normal, there is an inherent cost to the cell. As metabolic resources and enzyme activities are diverted to address redox

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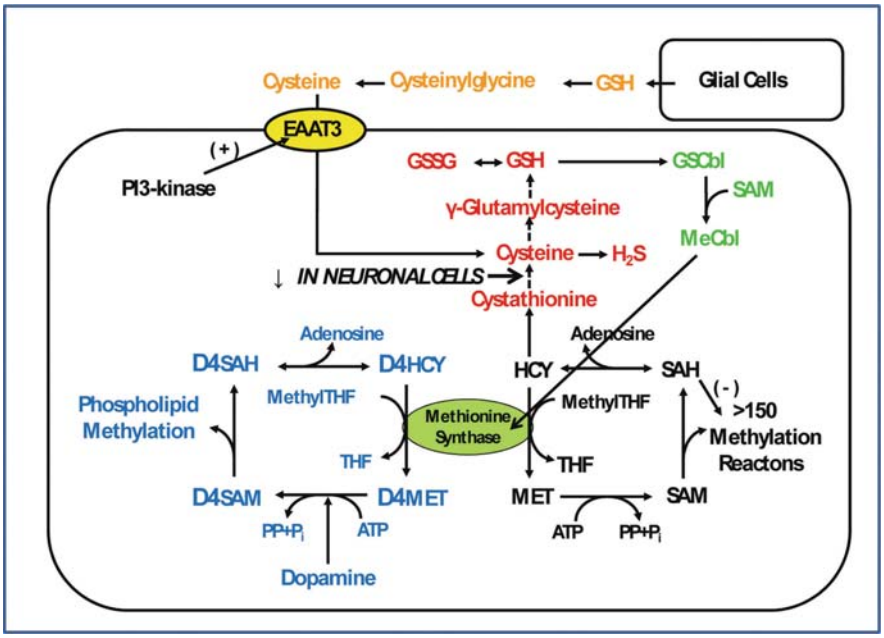

Fig. 1 GSH synthesis and methylation pathways in neuronal cells. Cysteine for GSH synthesis is provided by either uptake via EAAT3 or via transsulfuration of homocysteine (HCY), although transsulfuration is limited in neuronal cells, increasing the importance of uptake. Methionine synthase activity in neurons requires methylcobalamin (MeCbl), whose synthesis is GSH dependent. Dopamine-stimulated PLM is dependent upon methionine synthase activity. Methionine synthase activity determines levels of the methyl donor SAM and the methylation inhibitor SAH, affecting the efficiency of a large number of cellular methylation reactions.

needs, other specialized cellular activities are incrementally compromised during oxidative stress, resulting in a loss of functionality. All cells are affected by oxidative stress, so the particular loss of function depends upon the specialized nature of the cell (e.g., muscle, secretory, or neuronal). In the case of neurons, oxidative stress can cause a loss of cognitive abilities, including attention, learning, and memory (Dröge and Schipper, 2007).

Oxidative stress can be caused by a wide variety of factors, including inflammatory responses to infections or immune activation, exposure to heavy metals or toxic substances (Carpenter et al., 2002), and oxidative stress increases during the natural course of aging (Junqueira et al., 2007). When oxidative stress is induced by environmental exposures it represents a significant component of the toxicity syndrome, and most xenobiotics share the ability to cause oxidative stress. As a consequence, the effects of multiple exposures are additive at the level of oxidative stress. Metabolic changes associated with oxidative stress can be considered to be adaptive responses that increase prospects for survival during these stressful episodes.

Redox and Methylation

Methylation is the addition of a carbon atom to a molecule, usually causing a change in the function of the methylated molecule. For example, methylation of the neurotransmitter dopamine by catechol-*O*-methyltransferase renders it inactive. With only two exceptions, *S*-adenosylmethionine (SAM), an activated form of the essential amino acid methionine, is the methyl donor for each of the more than 150 methylation reactions, which regulate a large number of cellular functions. One exception is methylation of homocysteine (HCY) to methionine by the cobalamin (vitamin B_{12})-dependent enzyme methionine synthase, which utilizes 5-methyltetrahydrofolate (methylfolate) as the methyl donor, serving to complete the methionine cycle of methylation, as illustrated in Fig. 1 (lower right). Notably, HCY formation from *S*-adenosylhomocysteine (SAH) is reversible and, as a result, any decrease in methionine synthase activity will be reflected as an increase in both HCY and SAH. This is significant because SAH interferes with SAM-dependent methylation reactions, and a decrease in methionine synthase activity will decrease all of these reactions. Clearly methionine synthase exerts a powerful influence over cell function via its control over methylation.

As illustrated in Fig. 1, methionine synthase is positioned at the intersection between transsulfuration and methylation pathways. As a consequence, its level of activity exerts control over cellular redox status, since it determines the proportion of HCY that will be diverted toward cysteine and GSH synthesis. Methionine synthase activity is exceptionally sensitive to inhibition during oxidative stress, primarily because its cobalamin cofactor is easily oxidized (Liptak and Brunold, 2006). This allows methionine synthase to serve as a redox sensor, lowering its activity whenever the level of oxidation increases, until increased GSH synthesis brings the system back into balance. Electrophilic compounds, such as oxygen-containing xenobiotic metabolites, also react with cobalamin, inactivating the enzyme and increasing diversion of HCY toward GSH synthesis (Watson et al., 2004). Thus, methionine synthase is a sensor of both redox and xenobiotic status.

One of the most important roles of methylation is epigenetic regulation of gene expression through DNA methylation. Cytosine residues preceding guanine residues (i.e., CpG sites) frequently occur in promoter regions and their methylation promotes binding of a series of proteins that favor histone binding and inhibit transcription of adjacent genes (Miranda and Jones, 2007). Significant changes in patterns of DNA methylation occur during development, as genes are differentially turned off or on, and disruption of methylation by oxidative stress and impaired methionine synthase activity can therefore adversely affect development. Indeed, several neurodevelopmental disorders including Rett, Prader–Willi, and Angelman syndromes, as well as fragile X syndrome, have been linked to genetic defects involving DNA methylation (McConkie-Rosell et al., 1993; Wan et al., 1999; Thatcher et al., 2005). Oxidative stress associated with environmental exposure to xenotoxins may therefore mimic certain aspects of these disorders. Moreover, xenotoxin exposure can amplify the impact of methylation-related genetic risk factors, increasing the likelihood of developmental disorders.

Redox Regulation in the Brain

While all cells retain the basic features of sulfur metabolism, cell type-specific differences in their dynamic activity can be observed, with important consequences for redox- and methylation-related activities. These differences reflect the evolution of multicellular organisms, and the human brain, as a highly evolved organ, exhibits a unique pattern that supports its specialized function but also introduces higher vulnerability to oxidative stress. Understanding the unique pattern of sulfur metabolism in the human brain allows novel insights into autism and brain development.

In 1958 Tallan et al. measured cystathionine levels in brain tissue from different species and found the remarkable pattern shown in Fig. 2, which appears to reflect an evolutionary trend. Cystathionine was approximately 3-fold higher in human vs. monkey, 10-fold higher vs. rat, and up to 40-fold higher than other species. A comparison with other human tissues showed that brain levels were about 40-fold higher than liver, kidney, or muscle.

Cystathionine is the first intermediate metabolite in transsulfuration, formed from HCY and serine by cystathionine-β-synthase, a redox-sensitive, heme-containing enzyme (Banerjee et al., 2003), whose activity is lower in males vs. females (Vitvitsky et al., 2007). The higher levels of cystathionine in human brain reflect a strong diversion of HCY to transsulfuration (i.e., low methionine synthase activity and high cystathionine-β-synthase activity), in conjunction with a decreased conversion of cystathionine to cysteine. As illustrated in Fig. 1, this indicates impaired transsulfuration in human brain. Low transsulfuration activity relative to other tissues has been described in rat or mouse brain (Finkelstein, 1990), although a

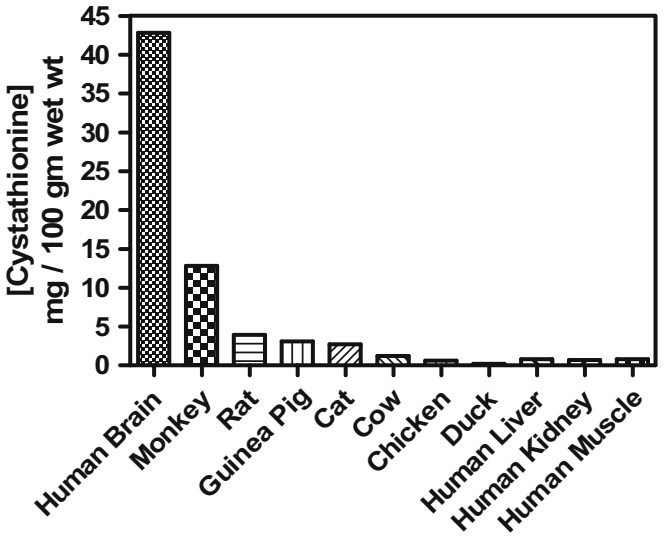

Fig. 2 Cystathionine levels are increased in human cortex. Levels of cystathionine were measured in cortex from different species and in different human tissues. Data are from Tallan et al. (1958)

recent study demonstrated that transsulfuration is at least partially functional in brain and helps to maintain GSH levels (Vitvitsky et al., 2006). Together these observations indicate that formation of cysteine via transsulfuration is restricted in brain, and particularly in human brain, increasing its vulnerability to oxidative stress.

An evolutionary perspective raises the important question: What are the implications and/or potential benefits of restricted transsulfuration? A key feature of neuronal cells is their ability to live for an entire lifespan without dividing, which is crucial for long-term retention of information in the form of synaptic connections within neural networks. If a neuron divides, it would lose all of its synapses and all of its information content, so a metabolic strategy must exist that keeps neurons out of the cell division cycle. Oxidative stress normally restricts neuronal division (Kruman, 2004), and limiting transsulfuration provides a mechanism by which neurons can indefinitely sustain a moderate level of oxidative stress. Thus, it can be proposed that the unique pattern of sulfur metabolism adopted by the brain may be critical for sustaining retention of information and for neuronal survival over the lifespan.

When cysteine synthesis via transsulfuration is restricted, the importance of cysteine uptake is greatly enhanced. Indeed the very survival of neurons is dependent upon the availability of extracellular cysteine and its regulated transport into the cell. Cysteine is made available to neurons via a multistep process involving glial cells, such as astrocytes. These cells take up cystine (i.e., oxidized cysteine), convert it to cysteine and GSH, and then export a significant portion of the GSH (Fig. 1). Extracellular peptidases successively remove glutamate and glycine, leaving cysteine available for uptake. Excitatory amino acid transporter-3 (EAAT3) is the primary uptake mechanism for cysteine in mature neurons (Aoyama et al., 2006), and its activity is increased by growth factors, via the PI3-kinase signaling mechanism (Sims et al., 2000), while activity is decreased by neurotoxins that lower GSH levels (Aoyama et al., 2008). Since cysteine uptake is so critical in neurons, growth factors exert a powerful influence over redox and methylation status via their ability to activate EAAT3. Growth factor release, occurring in association with increased neural activity, can utilize this mechanism to increase neuronal survival, augment neurite formation, or strengthen synapses, whereas oxidative stress will exert the opposite influence. Growth factors can also utilize redox and DNA methylation as a mechanism for epigenetic modulation of gene expression to promote differentiation during development (Feng et al., 2007).

Methionine synthase is composed of five structural domains that provide for binding of its substrate HCY, the methyl donor 5-methyltetrahydrofolate, cobalamin, and SAM (Fig. 4). In most tissues SAM is utilized to methylate oxidized cobalamin, in conjunction with electron donation by methionine synthase reductase, thereby restoring methylcobalamin and allowing resumption of activity. This mode of reactivation is required approximately every 100–1,000 turnovers, even under strictly anaerobic laboratory conditions (Bandarian et al., 2003). Under physiological conditions, oxidation of cobalamin is undoubtedly much more common, illustrating how vitamin B_{12} serves as a sensor of redox status. During oxidative stress, cobalamin is more frequently oxidized and more HCY is diverted toward cysteine and GSH synthesis.

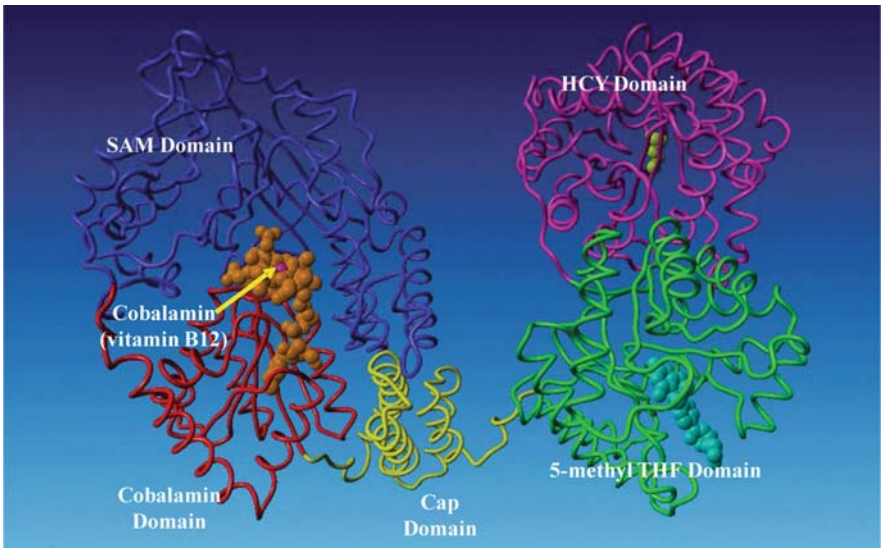

Fig. 3 Structural features of methionine synthase. Methionine synthase is comprised of five domains, which bind homocysteine (HCY), methylfolate (5-methyl THF), cobalamin, and *S*-adenosylmethionine (SAM). The Cap domain restricts oxidation of cobalamin in its vulnerable Cbl(I) state. Structures from *E.coli* (Bandarian et al., 2002; Dixon et al. 1996) and *T.maritima* (Evans et al. 2004) (PDB codes 1Q8J, 1K98 and 1MSK, respectively) were used to construct this composite model. An uncharacterized linker segment between the folate and cap domains is absent

Neuronal cells have a uniquely different strategy for reactivating methionine synthase, which is tightly dependent upon GSH status. Whereas SAM-dependent methylation of oxidized cobalamin occurs in most cell types, in neuronal cells oxidized cobalamin dissociates from the enzyme and is replaced by methylcobalamin, allowing reactivation. However, methylcobalamin synthesis proceeds through an intermediate step that requires GSH, so methionine synthase will remain inactive longer when GSH levels are below normal (Fig. 1). This relationship ensures that methylation activity in neurons, including dopamine-stimulated PLM described below, will be restricted under conditions of oxidative stress and also ensures that growth factor-induced cysteine uptake will exert a powerful influence over methylation. It is notable that D4 receptor stimulation leads to PI3-kinase activation (Zhen et al., 2001), and we have demonstrated that D4 receptor activation increases methionine synthase activity (Waly et al., 2004), implying that dopamine is capable of augmenting GSH levels in neuronal cells.

While four domains of methionine synthase bind reaction components (HCY, SAM, cobalamin, and methylfolate), the fifth domain, known as the Cap domain, hovers above cobalamin while it is in its readily oxidized Cob(I) state, limiting access of reactive oxygen species or electrophilic substances. As such, the Cap domain restricts inactivation of methionine synthase and consequently promotes methylation over transsulfuration. In rt-PCR studies using RNA from cultured human neuroblastoma cells, we found that the Cap sequence, corresponding to

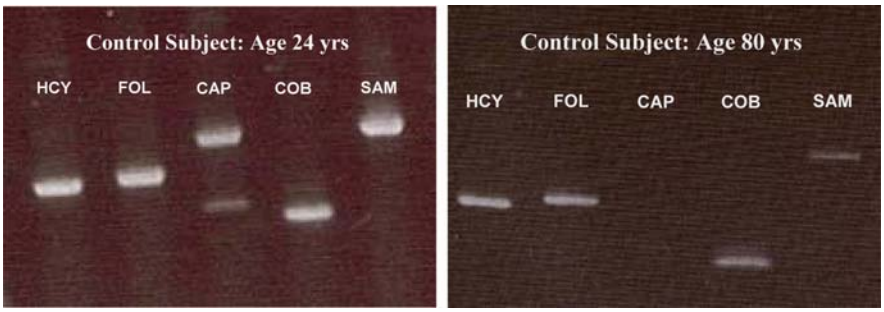

Fig. 4 Cap domain is absent in methionine synthase mRNA from aged subjects. rt-PCR was carried out with RNA from human cortex using primers for each of the five domains in methionine synthase. Note the presence of full (*upper band*) and partially spliced (*lower band*) products in mRNA from the 24-year-old subject

exons 19–21, was deleted from methionine synthase mRNA (Waly et al., 2008 C. Muratore, unpublished observation). As illustrated in Fig. 4, further examination of RNA from human cortex showed an age-dependent pattern, with the Cap domain being deleted in mRNA from all subjects over the age of 70 but present in mRNA from subjects under 30 years. Absence of the Cap domain, which occurs via alternative mRNA splicing, increases the vulnerability of methionine synthase to oxidation and augments GSH synthesis, representing a useful adaptive response to aging-associated increase in oxidative stress.

Dopamine-Stimulated Phospholipid Methylation

Dopamine utilizes five different G protein-coupled receptors (GPCRs) to exert its cellular effects. Among these, the D4 receptor subtype has a unique signaling activity by which it transfers folate-derived methyl groups to the headgroup of membrane phospholipids surrounding the receptor (Sharma et al., 1999). This process of phospholipid methylation (PLM) is activated by dopamine, based upon conformational movements of a methionine residue on transmembrane helix #6, located at the inner membrane surface where methylation takes place (Fig. 5). This methionine residue is not present in any other GPCR, making PLM a unique feature of the D4 receptor. While the functional role of dopamine-stimulated PLM remains to be fully elucidated, it provides a mechanism to alter membrane fluid properties in the receptor microenvironment, which can modulate the activity of membrane proteins. Computational models show that this mechanism can shift synchronized firing of neural networks to gamma frequency (30–80 Hz), which is observed during episodes of attention (Kuznetsova and Deth, 2008). Impaired neural synchrony is a consistent finding in autism, including diminished gamma frequency activity (Grice et al., 2001; Just et al., 2004).

The human D4 dopamine receptor exhibits a number of genetic variants affecting both transcription efficiency and protein sequence, making it one of the most variable human genes. Most prominent among these variants is a 48-bp variable

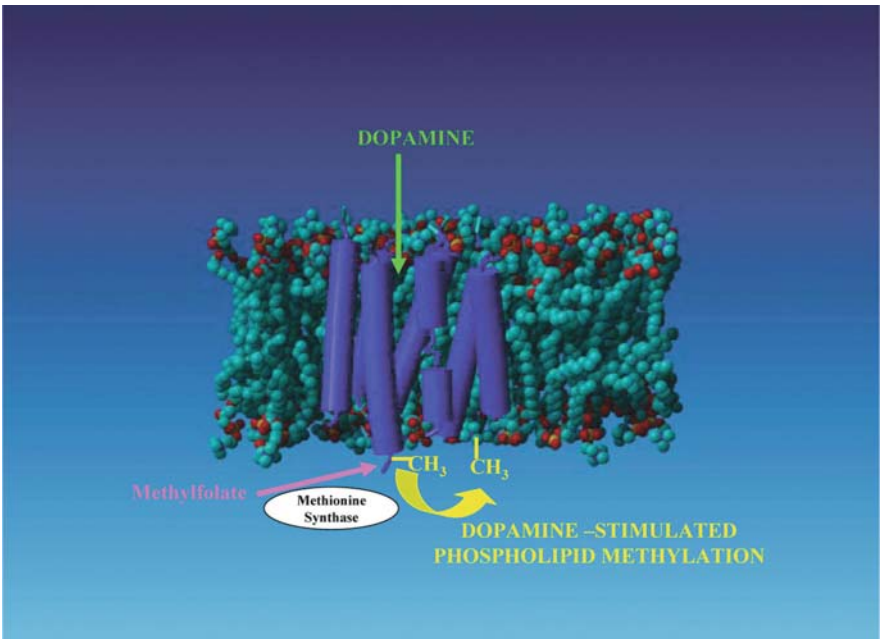

Fig. 5 D4 dopamine receptor-mediated phospholipid methylation. Dopamine occupation of the D4 receptor initiates transfer of a methyl group (CH_3) from an activated methionine residue to an adjacent phospholipid. A replacement methyl group is provided by methylfolate, via the action of methionine synthase

number tandem repeat, which varies from 2 to 11 repeats, with four repeats being most common in worldwide distribution (64% of alleles), followed by seven repeats (21%) and two (8%) (Chang et al., 1996). However, there are substantial fluctuations in the frequency of repeats among different ethnic groups and in different geographical regions. Presence of the seven-repeat allele has been linked to increased risk of attention-deficit hyperactivity disorder (ADHD) (Swanson et al., 2007), novelty-seeking personality traits (Munafò et al., 2008), obesity (Levitan et al., 2004), and infant attachment disorders (Lakatos et al., 2000); however, the frequency of seven-repeat variants in autistic subjects is similar to control (Grady et al., 2005).

Genetic analysis indicates that the seven-repeat form of the D4 receptor first arose about 40,000–50,000 years ago, a time period corresponding to the migration of humans out of Africa (Wang et al., 2004), and its distribution among different ethnic groups led to the suggestion that presence of the seven-repeat allele increased the propensity for exploration (Chang et al., 1996). Furthermore, the seven-repeat allele shows a pattern of positive selection (Ding et al., 2002), suggesting that it provides a beneficial function, although this function has yet to be identified. Its linkage to ADHD, whose incidence has increased in recent years, has led to the proposal that D4 receptor function, especially the seven-repeat receptor, is adversely affected by environmental exposures occurring in contemporary society (Swanson

et al., 2007). This perspective is reinforced by the fact that D4 receptor-mediated PLM is exquisitely sensitive to heavy metals and alcohol, reflecting the ability of these xenotoxins to promote oxidative stress (Waly et al., 2004). Interestingly, the power of increased gamma frequency synchronization during attention is greater in individuals who possess one or more seven-repeat alleles (Demiralp et al., 2007), raising the possibility that increased exposure to environmental xenotoxins may be interfering with this role of dopamine. Gamma synchrony promotes coordination between brain regions, which is especially important for complex tasks. Recently evolved human abilities, such as language and social awareness, may be particularly vulnerable.

As illustrated in Fig. 1, dopamine-stimulated PLM is dependent upon a supply of folate-derived methyl groups provided via methionine synthase activity. Indeed, while dietary methionine can provide an ongoing source of methyl groups to support non-D4 receptor methylation, dopamine-stimulated PLM is completely dependent upon the availability of methylfolate and ongoing methionine synthase activity, including cobalamin status. Functional correlates of dopamine-stimulated PLM, such as the level of gamma synchronization during attention, are likewise dependent upon these factors. In a study of neurodevelopmental toxins using cultured human neuroblastoma cells, we found that dopamine-stimulated PLM and methionine synthase activity were significantly inhibited at concentrations of mercury, lead, aluminum, and thimerosal alcohol that are typical of plasma concentrations encountered during human exposure (Waly et al., 2004). Subsequent studies showed that inhibition was due to lower levels of GSH, which interfered with synthesis of the active form of cobalamin, methylcobalamin, also known as methyl-B_{12}, which is required for reactivation of methionine synthase whenever its activity is interrupted by oxidation (M. Waly, unpublished observation).

Oxidative Stress and Neuroinflammation in Autism

A substantial and growing body of evidence indicates that oxidative stress and neuroinflammation are closely associated with autism and are likely to be critical factors in causing the disorder (for reviews see McGinnis, 2004; Kern and Jones, 2006; Chauhan and Chauhan, 2006; Deth et al., 2008). Plasma levels of GSH, as well as methionine cycle and transsulfuration metabolites, are abnormal in autistic individuals (James et al., 2004; James et al., 2006; Geier and Geier, 2006a; Paşca et al. 2008). Adenosine and SAH levels are increased while HCY, methionine, and SAM levels are low, consistent with decreased methionine synthase activity and increased cystathionine-beta-synthase activity, while the SAM/SAH ratio is significantly reduced, indicating impaired methylation capacity. Cystathionine, cysteine, and GSH levels are decreased, along with the GSH/GSSG ratio, reflecting increased oxidative stress. Elevated HCY levels have also been reported in autism (Pasca et al., 2006). Supplementation with a combination of betaine (trimethylglycine) and folinic acid (5-formyl THF) normalized methionine cycle metabolites,

but transsulfuration metabolites remained abnormal (James et al., 2004). Upon further addition of methylcobalamin, levels of all metabolites, as well as SAM/SAH and GSH/GSSG ratios, returned to normal. These abnormal metabolic profiles are now confirmed and they represent a critically important clue to the origins of autism.

Oxidative stress in autism is associated with increased plasma levels of malondialdehyde, urinary levels of fatty acid, and lipid peroxidation biomarkers (Chouhan et al., 2004; Zoroglu et al., 2004; Ming et al., 2005; Yao et al., 2006). Elevated levels of inflammatory cytokines and evidence of microglial activation are observed in postmortem brain sections indicating the presence of neuroinflammation (Vargas et al., 2005). Microglial cells monitor the local environment and provide a macrophage-like function in the brain, releasing pro-inflammatory substances upon activation. In addition, microglia take up organic mercury and convert it to the more toxic inorganic mercury (Charleston et al., 1995), and in primate cortex, chronic methylmercury exposure leads to a large increase in activated microglia (Charleston et al., 1994). Heavy metals can therefore cause oxidative stress in neurons not only by their direct influence on sulfur metabolism but also by promoting microglia-based neuroinflammation.

Oxidation of cobalamin during oxidative insults provides a short-term mechanism to augment transsulfuration and GSH synthesis, but chronic neuroinflammation and prolonged oxidative stress can activate additional, longer term adaptive responses to restrict methionine synthase activity. These could include decreased transcription of the methionine synthase gene, decreased translation of its mRNA, increased degradation of the protein, and/or decreased cellular uptake of cobalamin or folic acid cofactors. To evaluate brain methionine synthase status in autism, we carried out quantitative RT-PCR using cortex RNA samples from autistic subjects and age-matched neurotypical control subjects. These RNA samples were derived from the same tissues in which Vargas et al. (2005) described the presence of neuroinflammation. We utilized primers directed against both cobalamin-binding and cap domains of methionine synthase, and in both cases the level of mRNA was significantly lower in autism samples, amounting to a decrease of two to threefold (Fig. 6). As outlined above, lower activity of methionine synthase increases HCY diversion to transsulfuration and GSH synthesis; thus, we interpret the reduction in methionine synthase mRNA as an adaptive response to oxidative stress and neuroinflammation. This finding confirms impaired methylation in the brain during autism, and in particular it indicates that the supply of methyl groups for dopamine-stimulated PLM activity will be reduced.

Genes play a major role in autism, as reflected by high concordance rates between monozygotic twins (Smalley et al., 1988), but this does not necessarily imply that genetic defects are the cause of autism. A study of single-nucleotide polymorphisms (SNPs) in genes affecting redox and methylation found that autistic subjects had a significantly higher prevalence of risk-inducing SNPs (James et al., 2006). These genes included transcobalamin II and the reduced folate carrier, which transport cobalamin and folate into cells, as well as methionine synthase reductase, which is responsible for reduction of oxidized cobalamin, and catecholamine-*O*-methyltransferase (COMT), which inactivates dopamine and norepinephrine.

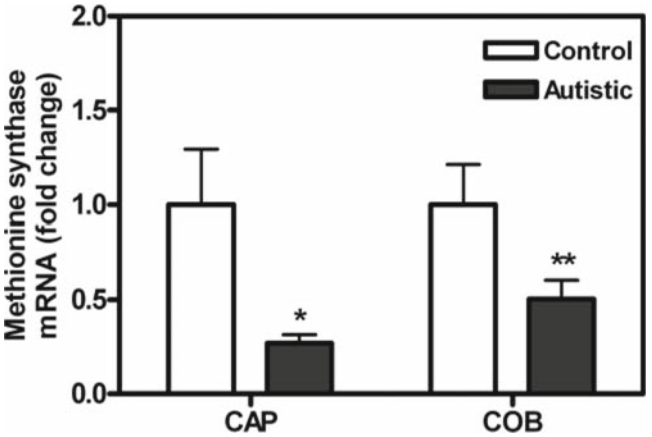

Fig. 6 Methionine synthase mRNA levels in human cortex are reduced in autism. RNA samples from autistic and non-autistic subjects were probed using qRT-PCR with specific primers to the CAP and COB domains of methionine synthase. $n = 11$ for each group ($^{*}p<0.05$ compared to control for the CAP primer set; $^{**}p<0.05$ compared to control for the COB primer set)

Importantly, these SNPs are normal features of human genes, contributing to individual variability in the metabolic processes they control. When combinations of SNPs were evaluated, an odds ratio up to 7-fold was found, providing a clear example of how genetic risk factors might contribute to autism, but at the same time not necessarily representing the critical causative factor.

The Autism/Vaccination Controversy

The recent dramatic increase in autism prevalence strongly suggests that one or more environmental toxins may be a causative factor, and it has been proposed that the ethylmercury derivative thimerosal, used as a preservative in vaccines, might be such a factor (Bernard et al., 2002). Most, but not all, (Geier and Geier, 2006b; Gallagher and Goodman, 2008) epidemiologic studies have failed to find an association between thimerosal and autism (Hviid et al., 2003; Verstraeten et al., 2003; Andrews et al., 2004), and autism rates continue to rise despite its removal from most vaccines (Schechter and Grether, 2008), but this highly controversial proposal remains a lightning rod within the autism community.

Our laboratory was the first to demonstrate the ability of thimerosal to potently inhibit methionine synthase activity in cultured human neuronal cells (Waly et al., 2004). Subsequent research has shown that inhibition results from a reduction in GSH levels and impaired methylcobalamin synthesis, under conditions where methionine synthase activity is absolutely dependent upon methylcobalamin (M. Waly, unpublished observation). The fact that autistic subjects exhibit lower GSH levels and lower methionine synthase activity lends credence to the mercury

hypothesis, but these effects are not unique to mercury and can be produced by other xenotoxins (Deth et al., 2008).

Since autism rates are uniformly increased across the USA, exposure to the putative offending agent(s) must be widespread. Pesticides, herbicides, flame retardants, solvents, and plasticizing agents are among the vast number of chemical agents that interact directly or indirectly with redox and methylation pathways. Aluminum, which is still included in many vaccines as an adjuvant that improves the immune response, merits further evaluation. Aluminum, mercury, and other heavy metals can augment antibody formation by increasing Th2 helper cell activity (Kidd, 2003), an action that can be linked to their ability to lower GSH levels in cells (Agrawal et al., 2007). Aluminum inhibits methionine synthase and lowers GSH levels in human neuronal cells with high potency, similar to the effects of mercury (Waly et al., 2004).

Future Directions

The unique ability of dopamine to alter membrane properties via D4 receptor-mediated PLM and the vulnerability of this process to oxidative stress implies that its impairment might be a significant factor in autism. However, further studies are needed to confirm the physiologic role of dopamine-stimulated PLM in attention and neuronal synchronization in intact animal models. Specifically, it would be valuable to assess the impact of oxidative stress on attention and neuronal synchrony. While some features of redox and methylation regulation are human specific, these studies could shed new light on the metabolic events underlying autism. Moreover, there is a critical need for suitable animal models capable of evaluating candidate xenotoxins for their influence on redox and methylation status in the brain. Rising autism rates elevate the importance of such studies to the highest public health priority.

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Neuroligins and Neurexins: Synaptic Bridges Implicated in Autism

Craig M. Powell and Antony A. Boucard

Introduction

Loss-of-function mutations in members of the Neuroligin (NL) family of trans-synaptic cell adhesion molecules have been implicated in human autism and mental retardation (Chih et al., 2004; Comoletti et al., 2004; Jamain et al., 2003; Laumonnier et al., 2004; Yan et al., 2005). At the time of writing, neuroligin mutations represent owe of only a few independently replicated, functional, genetic mutation links to idiopathic autism not associated with a broader neuropsychiatric syndrome (Chih et al., 2004; Comoletti et al., 2004; Jamain et al., 2003; Laumonnier et al., 2004; Yan et al., 2005).

More recently, the presynaptic binding partner of neuroligin, neurexin-1, has also been implicated in human autism. An internal deletion in the gene encoding neurexin-1, a presynaptic cell adhesion molecule that binds postsynaptic neuroligins, has been linked to ASD in one family (Szatmari et al., 2007; Feng et al., 2006). These findings firmly implicate a trans-synaptic neuroligin/neurexin cell adhesion bridge in rare genetic causes of autism.

In addition, three different nonsense mutations in the gene encoding the post-synaptic scaffolding protein, *Shank3* (also known as *ProSAP2*), were found in three separate patients with ASD (Durand et al., 2007). Shank3 was identified as an intracellular binding partner of neuroligins by yeast two-hybrid screen (Meyer et al., 2004), though this interaction remains to be confirmed. Furthermore, children affected by Phelan-McDermid syndrome (22q13 Deletion Syndrome) have autistic features along with loss of the *shank3* gene (Bonaglia et al., 2001).

Mutations in members of the neuroligin and neurexin families of trans-synaptic cell adhesion molecules have been implicated in human autism and mental retardation (Chih et al., 2004; Comoletti et al., 2004; Jamain et al., 2003; Laumonnier et al., 2004; Yan et al., 2005). The phenotypic variability from autism spectrum

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to mental retardation in humans with neuroligin and neurexin mutations mirrors to some extent the phenotypic variation within autism spectrum disorders. Animal models of autism not associated with broader neuropsychiatric syndromes have been severely limited, but these human genetic findings provide a compelling rationale for developing bona fide mouse models of at least a subtype of human autism/mental retardation. Furthermore, these findings provide the opportunity to understand at a molecular, cellular, and microcircuit level the pathogenesis of a subset of autism spectrum disorders. In this chapter, we discuss neuroligin and neurexin function, genetic link to autism, mouse models, and potential for understanding the pathogenesis and ultimately treatment of autism spectrum disorders linked to these genes.

Neuroligins and Neurexin-1 as Candidate Autism Genes

Autism is known to have a strong genetic component (Folstein and Rosen-Sheidley, 2001; Gillberg, 1998; Veenstra-Vanderweele et al., 2003; Veenstra-VanderWeele and Cook, 2004), and mutations in NL3 and NL4 are associated with autism/mental retardation in humans (Jamain et al., 2003; Laumonnier et al., 2004). Specifically, one study identified two Swedish families with autism or Asperger syndrome linked to either a frameshift mutation in NL4 (leading to premature termination of the protein before the transmembrane domain) or to an arginine to cysteine (R451C) mutation in NL3 (Jamain et al., 2003). A second study identified a 2-base pair deletion in NL4 leading to a premature stop codon in a French family affected by X-linked mental retardation with or without autism (Laumonnier et al., 2004). A third study of 148 unrelated autism patients found four different missense mutations in NL4 via direct sequencing (Yan et al., 2005). These data suggest that mutations in NL3 and NL4 are linked to autism, though they may be responsible for only a small portion of the cases (Gauthier et al., 2005; Vincent et al., 2004; Ylisaukko-oja et al., 2005).

NL mutations associated with autism may lead to loss of function. Some of the mutations identified lead to premature termination of the NL protein prior to the transmembrane domain, implying a loss of function (Jamain et al., 2003; Laumonnier et al., 2004; Talebizadeh et al., 2004; Yan et al., 2005). The NL3 R451C and NL4 D396X mutations have been studied extensively in vitro and are known to lead to functional inactivation, likely via retention in the endoplasmic reticulum (Chih et al., 2004; Chubykin et al., 2005). The recently published NL3 point mutant mouse model of autism, however, suggests that this mutation may act in part via a gain-of-function mechanism (Tabuchi et al., 2007). These findings suggest that both the loss of neuroligin function and gain-of-function mutations may result in an autism-like phenotype.

Neurexin-1, a trans-synaptic cell adhesion molecule that binds to neuroligins, has also been implicated in ASD (Szatmari et al., 2007; Feng et al., 2006). Briefly, large internal deletions in neurexin-1 coding exons were found in two affected siblings

in a very large genetic screen of autism families with multiple affected children (Szatmari et al., 2007). This finding extends the potential role of neuroligin 3 and neuroligin 4 mutations in autism to their presynaptic binding partner neurexin-1.

In addition to neurexins, shank 3, a postsynaptic scaffolding protein that may interact with neuroligins (Meyer et al., 2004), has been found to be linked to ASD (Durand et al., 2007). Three different nonsense mutations were found in three separate cases of autism using a candidate gene approach. These mutations clearly associated with affected individuals within families. Thus, it appears that the neurexin-1/neuroligin-3/shank3 signaling complex is linked to a small subset of genetic cases of ASD.

Neurexins: Discovery, Domain Structure, and Brain Distribution

Neurexins (Nrx) were first discovered in a search for the potential targets of a spider venom neurotoxin named α-latrotoxin which induces robust neurotransmitter release (Tzeng and Siekevitz, 1978; Ushkaryov et al., 1992). Action of the toxin was shown to be both dependent and independent of the presence of extracellular Ca^{2+}. Affinity chromatography using immobilized α-latrotoxin led to the purification of neurexins from brain extracts through an interaction depending on the presence of Ca^{2+} (Ushkaryov et al., 1992). This Ca^{2+}-dependent high-affinity interaction between the toxin and the neurexin not only gave a molecular basis for the action of latrotoxin but also led to the hypothesis that neurexin could be a part of the presynaptic machinery, a hypothesis that was later supported by immunoelectron microscopy performed on cultured hippocampal neurons (Taniguchi et al., 2007). Sequencing of the neurexins revealed that they constitute type 1 transmembrane proteins composed of an extracellular domain, a single transmembrane domain, and a cytoplasmic tail harboring a class II PDZ (PSD95/Dlg/Zo-1) binding domain (Ushkaryov et al., 1992) (Fig. 1).

Vertebrate genomes contain three neurexin genes, neurexin 1, 2, 3, each one generating mRNA transcripts for a long form (α-neurexin) and a short form (β-neurexin) due to the presence of two alternative promoters (Ushkaryov et al., 1992). The diversity of neurexin proteins is also augmented by the presence of multiple alternative splice sites on their extracellular region: five sites for α-neurexin (SS1-5) and two sites for β-neurexin (SS4-5) (Tabuchi and Sudhof, 2002). The α-neurexin extracellular region contains multiple cell adhesion motifs bearing some level of similarity with laminin-G, the sex hormone binding protein, and to the epidermal growth factor (LNS domains) while β-neurexins contain only one LNS domain which is shared with α-neurexin. In situ hybridization suggests a partly overlapping/partly differential distribution of neurexin splice variant expression (Ullrich et al., 1995). Thus, the extracellular domains of α- and β-neurexin differ while their transmembrane and cytoplasmic regions are identical. Described as presumptive presynaptic cell adhesion molecules, neurexins were thought to bind an as yet unknown postsynaptic cell adhesion molecule later found to be neuroligins.

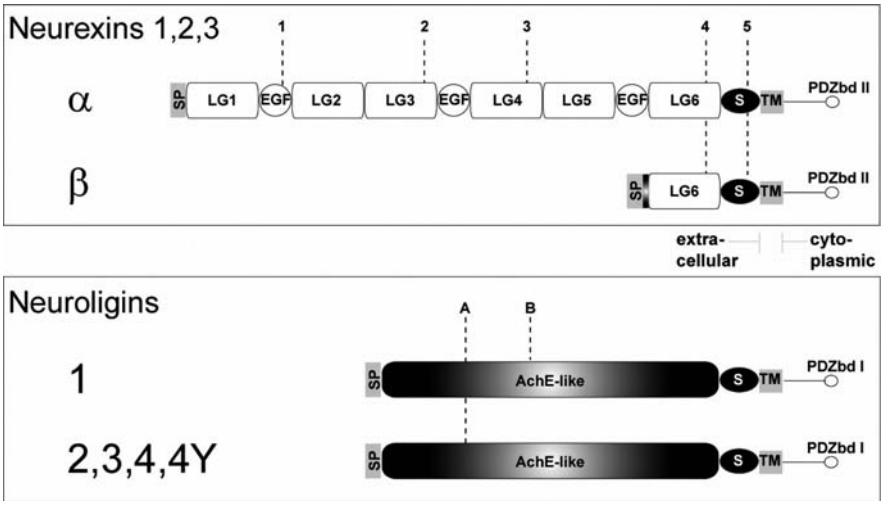

Fig. 1 Domain structure of neurexins and neuroligins. *Top* shows the domain organization of α- and β-neurexins: SP, signal peptide; LG1–6, laminin-G domain 1–6 (also called LNS domains for Laminin/Neurexin/Sex hormone binding domains); EGF, epidermal growth factor-like domain; S, carbohydrate attachment site and stalk region; TM, transmembrane region; PDZbd II, class II PDZ binding domain. *Bottom* shows the domain organization of neuroligins: AchE, acetylcholinesterase-like domain; S, carbohydrate attachment site and stalk region; TM, transmembrane region; PDZbd I, class I PDZ binding domain. Extracellular domain and cytoplasmic domain are indicated. Sites for alternative splicing are represented with numbers for neurexins (1–5) and letters for neuroligins (**A** and **B**) over *dashed lines*. Schematized are neuroligins from the human genome; only four neuroligins have been identified in mouse genome (NL1 2, 3, and 4∗). Note that splice insert B is only present for neuroligin 1

Neuroligins: Discovery, Domain Structure, and Brain Distribution

Neuroligins (NL) were first discovered as a ligand for β-neurexins using affinity chromatography on immobilized neurexin 1β (Ichtchenko et al., 1995). More recently binding of neuroligin to α-neurexin was also described, thereby unifying the binding functions of both neurexins (Boucard et al., 2005). Like neurexins, NLs are type 1 transmembrane proteins. They contain a large extracellular domain, one transmembrane region, and a cytoplasmic region containing a class I PDZ binding domain (Ichtchenko et al., 1995; Ichtchenko et al., 1996) (Fig. 1). Five human NL genes are known (*NL1, 2, 3, 4, and 4Y*), while in rodents, only four have been identified to date (*NL 1, 2, 3, and 4**) (Bolliger et al., 2001; Bolliger et al., 2008; Jamain et al., 2008). Both NL1 and NL2 are strongly expressed in many brain regions, including hippocampus and neocortex. NL3 is expressed at comparable levels throughout the brain including cortical structures (T. Südhof, unpublished communication; Varoqueaux et al., 2006). Their extracellular domains show high homology to acetylcholinesterase but is presumed to lack enzymatic activity due to the absence of key residues important for catalytic activity (Scholl and Scheiffele,

2003). Like neurexins, the NL extracellular domain contains sites for alternative splicing, splice site A and B (SSA and SSB) (Ichtchenko et al., 1995; Ichtchenko et al., 1996). While an alternative splice insert is found in SSA for all neuroligins, the SSB insert seems to be specific for NL1 (Comoletti et al., 2006).

Role of Alternative Splicing in Neurexin/Neuroligin Interaction

In both cases alternative splicing not only increases the diversity but also regulates binding between neuroligins and neurexins (Fig. 2). Indeed the neurexin SS4 insert seems to modulate binding to neuroligins, whereas the neuroligin SSB insert strictly

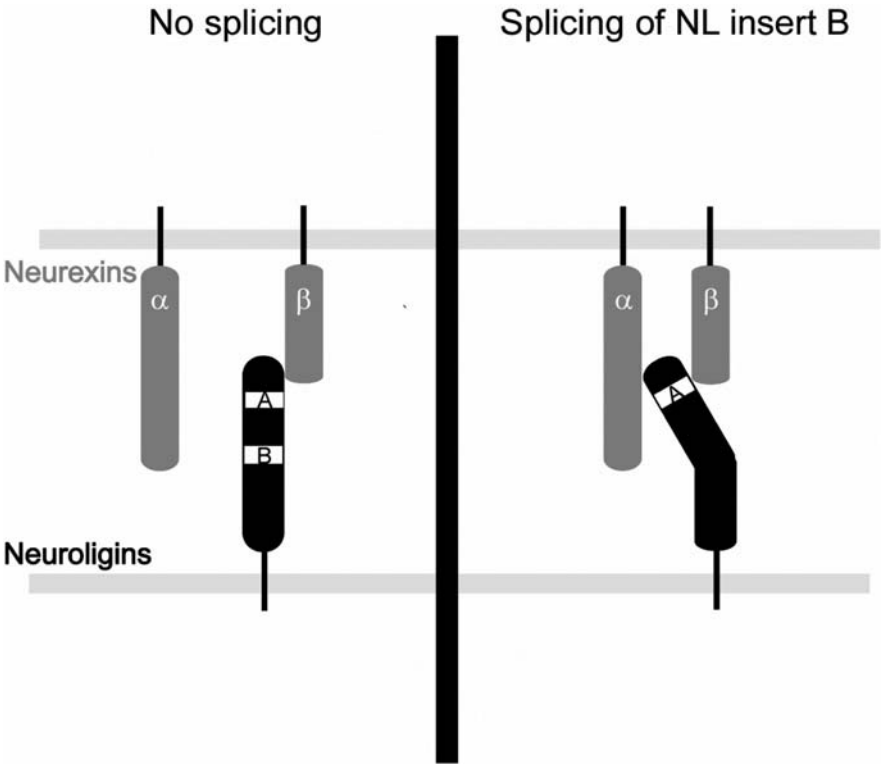

Fig. 2 Alternative splicing of neuroligins governs interaction with α- and β-neurexins. Neuroligins (NL) and neurexins are presumed to be on opposing membranes at the synapse, i.e., presynaptic membrane for neurexin and postsynaptic membrane for neuroligins. Neuroligin 1 containing inserts in both splice sites **A** and **B** interacts exclusively with β-neurexins (whether β-neurexins contain an insert in splice site 4 or not). However, neuroligins lacking an insert in splice site B (which is the case for all neuroligins; neuroligin 1 being the only one for which splice variants can contain an insert in splice site B) interact with both α- and β-neurexins

allows binding to either α- or β-neurexins (Boucard et al., 2005). The striking effect of neuroligin SSB insert on binding relies on the presence of glycosylation that hinders binding when the insert is present but allows promiscuous binding when the insert is absent. Crystal structures of the neurexin/neuroligin complex also provide support for the role of SS4 and SSB in binding: both splice sites are located at the binding interface between the two molecules (Arac et al., 2007; Chen et al., 2008; Fabrichny et al., 2007). Also the observation that crystals made using neurexin containing the SS4 insert and neuroligin display high instability would suggest that this insert may weaken the intermolecular interaction (Koehnke et al., 2008; Shen et al., 2008). Although neuroligins seem to appear as dimers in a complex with neurexin, alternative splice sites do not span the dimerization interface and therefore should not alter neuroligin dimer formation (Arac et al., 2007; Chen et al., 1995; Fabrichny et al., 2007). The precise role of the neuroligin–neurexin interaction is unknown but may be involved in either initial contact of pre- and postsynaptic elements or the post hoc stabilization of this contact (Levinson et al., 2005; Sudhof, 2001). One possible mechanism of action is that, upon binding, postsynaptic neuroligins induce clustering of presynaptic neurexins or vice versa and therefore lead to the recruitment of intracellular adaptor proteins.

Involvement of Neurexin/Neuroligin in Synapse Formation or Validation

The heterophilic interaction described by neurexin/neuroligin pairing prompted the hypothesis that they could serve as cell adhesion molecules involved in synapse formation, a hypothesis relying on different observations. First, neurexins and neuroligins interact with different synaptic proteins through their PDZ binding domain, providing a potential mechanism for the asymmetry of synapses: CASK and Mint for neurexins and PSD95 for neuroligins (Biederer and Sudhof, 2000; Butz et al., 1998; Irie et al., 1997). Second, heterologous cells separately overexpressing neurexins and neuroligins acquire the ability to aggregate when mixed together, thereby supporting their role in cell adhesion mechanisms (Nguyen and Sudhof, 1997). Third, both neurexin and neuroligin developmental expression correlates with the peak of synaptogenesis (Song et al., 1999; Sugita et al., 2001). Fourth, biochemical evidence suggests that they are both enriched at synaptic plasma membranes (Berninghausen et al., 2007; Song et al., 1999). Finally, neuroligins and neurexins when overexpressed in heterologous cells initiate synaptic specialization of contacting neurons in a coculture system developed by Scheiffele et al.; while postsynaptic markers are recruited at contact sites with neurexin expressing cells, presynaptic elements are favored for neuroligin expressing cells (Dean et al., 2003; Graf et al., 2004; Scheiffele et al., 2000). The relevance of these cultured neuronal systems to actual mechanisms in developing neurons in vivo, however, remains limited.

Neuroligins are differentially localized to excitatory or inhibitory synapses. NL1, for example, is preferentially found at excitatory synapses in vivo (Song et al., 1999). Neuroligin 2, on the other hand, localizes largely to inhibitory synapses (Varoqueaux et al., 2004). Neuroligin 3 may be found at both excitatory and inhibitory synapses in vivo (Budreck and Scheiffele, 2007). The intriguing segregation of NL isoforms adjacent to synaptic specializations has raised the question of whether extracellular or intracellular domains are responsible for their targeting. While NL differential localization has been recently attributed to their differential binding and molecular ratio to PSD95 (Prange et al., 2004), other studies suggest that neither the extracellular domain (Dresbach et al., 2004) nor the cytoplasmic PDZ binding domain (Chih et al., 2005; Dresbach et al., 2004) of NL govern their neuronal localization. This suggests that NLs might have other synaptic ligands than the neurexins and PSD95 that would explain a differential role in synaptogenesis for their various isoforms.

NLs have been shown not only to affect neuronal morphology but also to influence synaptic physiology. Presumably, NLs are linked to the postsynaptic NMDA receptor signaling machinery through their interaction with PSD95. Studies in cultured neurons indicate that overexpressing NLs can indeed control the NMDA/AMPA ratio and therefore elicit changes in excitatory versus inhibitory currents in neurotransmission (Chih et al., 2005; Chubykin et al., 2005; Dean et al., 2003; Graf et al., 2004; Levinson et al., 2005; Levinson and El-Husseini, 2005a, b; Nam and Chen, 2005; Prange et al., 2004; Scheiffele et al., 2000). Furthermore, knockdown studies of NLs using RNA interference (RNAi) technology resulted in a change of both excitatory and inhibitory synaptic transmission (Chih et al., 2005), suggesting that NLs are involved in balancing or validating both types of synapses. This validation process mediated by NLs seems to require neuronal activity since chronic pharmacological treatments antagonizing NMDA or AMPA receptor functions abolish the NL effects following overexpression in hippocampal neurons (Chubykin et al., 2007).

Although cell culture assays have provided useful insights about the potential role of neurexin/neuroligin, animal models have been created to explore their function in vivo. In the case of neurexins, only the α-neurexin knockout (KO) mice have been investigated using this technology (Missler et al., 2003). The α-neurexin triple KO mice show perinatal lethality presumably due to reported respiratory problems. They also exhibit a reduction in evoked and spontaneous neurotransmission at inhibitory and excitatory synapses as well as a defect in Ca^{2+} channel function. However, despite their presumed contribution to synapse formation, no abnormalities in synaptic ultrastructure were observed in these mice probably due to redundancy given that β-neurexins are still expressed. In the case of the neuroligins, NL1/2/3 triple KO mice have been published and exhibit relatively normal synaptogenesis (Varoqueaux et al., 2006). Synapse morphology by electron microscopy in hippocampal neuronal cultures is normal. Density of excitatory and inhibitory synapses in cortical and hippocampal regions appears normal (unpublished communication, Südhof, T.C.). Indeed, only in the respiratory control nuclei of the brain stem alterations in both inhibitory and excitatory synaptic function have been

demonstrated (Varoqueaux et al., 2006). These in vivo studies indicate that synapse formation does not strictly require neuroligins 1–3, though NL4 may compensate in this setting.

Alterations in synapse function, synaptogenesis, and ratio of E/I synapses have been postulated as mechanisms for human autism, making neuroligin dysfunction a particularly interesting candidate for animal models (Rubenstein and Merzenich, 2003; Zoghbi, 2003).

Autism Animal Model Based on Neuroligin 3 Mutation

Although identification of specific mutations in neuroligins and neurexins represent a major advance in autism research, identification of a gene or genetic mutation is only the beginning of an arduous journey to understand the underlying pathophysiology of autism spectrum disorders. In spite of vast amounts of knowledge regarding neurexin and neuroligin, we still do not fully understand their function in vivo. More importantly for autism, we have little understanding of how mutations in neuroligins or neurexins could lead to altered behaviors that represent autism spectrum symptoms. The effort to answer these important questions begins with genetically accurate animal models in which we can generate and test specific hypotheses and understand how mutations alter brain structure function and how this might relate to autism-related behavioral abnormalities in the model. The recent publication of a genetically modified mouse in which the normal neuroligin 3 gene is replaced with a neuroligin 3 gene containing a single DNA base pair change that occurs in humans in association with autism or Asperger's is just such an animal model (Tabuchi et al., 2007).

The NL3 R451C knockin mouse model is arguably the first genetically accurate mouse model of ASD not associated with a broader neuropsychiatric syndrome. It is "genetically accurate" because it recapitulates exactly the point mutation in NL3 that is linked to ASD that leads to a single amino acid change from an arginine to cysteine at amino acid number 451 (NL3 R451C). The caveat "not associate with a broader neuropsychiatric syndrome" is necessary because genetically accurate models of neuropsychiatric syndromes such as Fragile X (Garber et al., 2008; Penagarikano et al., 2007), Rett syndrome (Chahrour and Zoghbi, 2007; Zoghbi, 2005), and others have already been made and characterized. NL3 R451C mutations do not appear to be associated with a broader neuropsychiatric syndrome, though only a single family was described with two affected individuals, one with autism and mental retardation and the other with Asperger's (Jamain et al., 2003).

The NL3 R451C mouse model of autism exhibits autism-related behavioral abnormalities and enhanced inhibitory synaptic transmission in the cortex as outlined in more detail below.

NL3 R451C KI mice exhibit a selective deficit in social interaction/approach behavior (Fig. 3) but enhanced spatial cognitive function (Fig. 4) (Tabuchi et al., 2007). R451C KI mice showed no change in the time of interaction with a novel

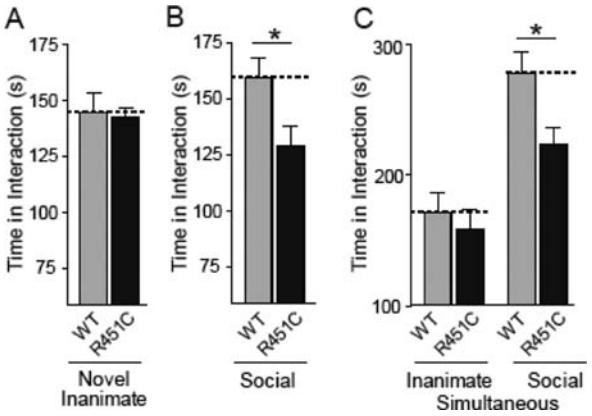

Fig. 3 Impaired social interaction behaviors in neuroligin 3 R451C KI mice. (**A**) Interacting time of individual wild-type and R451C KI mice exposed to a novel inanimate object in an unfamiliar cage (5 min). (**B**) Interacting time of mice that are exposed to an unfamiliar immobilized target mouse in a now familiar cage (5 min; procedure immediately follows A). (**C**) Interacting time of mice that are exposed simultaneously to a novel inanimate object and a novel, caged target mouse. All data shown are means ± SEMs; $n = 19$ male littermate pairs; only statistically significant differences between wild-type and R451C KI mice are specifically identified in the figure ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$ by *t*-test or two-way ANOVA); for detailed statistical analysis see Tabuchi et al. (2007). From Tabuchi et al. (2007)

inanimate object, but a significant decrease in interaction with a novel caged adult target mouse compared to wild-type littermate controls, indicating a social interaction deficit (Fig. 3A). Similarly, in a test for social versus inanimate preference, R451C KI mice spent significantly less time interacting with a social target than the wild-type littermate controls (Fig. 3B). In agreement with a selective effect on social behavior, R451C KI mice spent the same amount of time interacting with an inanimate target as controls during this task.

To examine whether the selective decrease in social interactions in R451C KI mice is associated with a gain or a loss of other cognitive abilities, spatial learning and memory were examined in R451C KI mice using the Morris water maze. R451C KI mice learned to locate and mount a visible platform as well as wild-type littermate control mice, indicating that basic neurologic functions required for swimming, vision, etc., were intact. When the platform was hidden, the R451C KI mice exhibited a significantly enhanced ability to locate the platform (Fig. 4A) and required fewer days of training to learn the location of the platform (Fig. 4B). During the probe trial 24 hours after the seventh day of training, both wild-type and R451C KI mice displayed a significant preference for the target versus the opposite quadrant, but the R451C KI mice crossed the precise former location of the target platform almost twice as often as their wild-type littermate controls (Fig. 4C).

To ensure that an increase in number of target location crossings in the R451C KI mice in the probe trial was due to enhanced spatial memory rather than perseveration, the location of the platform was reversed and the same cohort of mice was

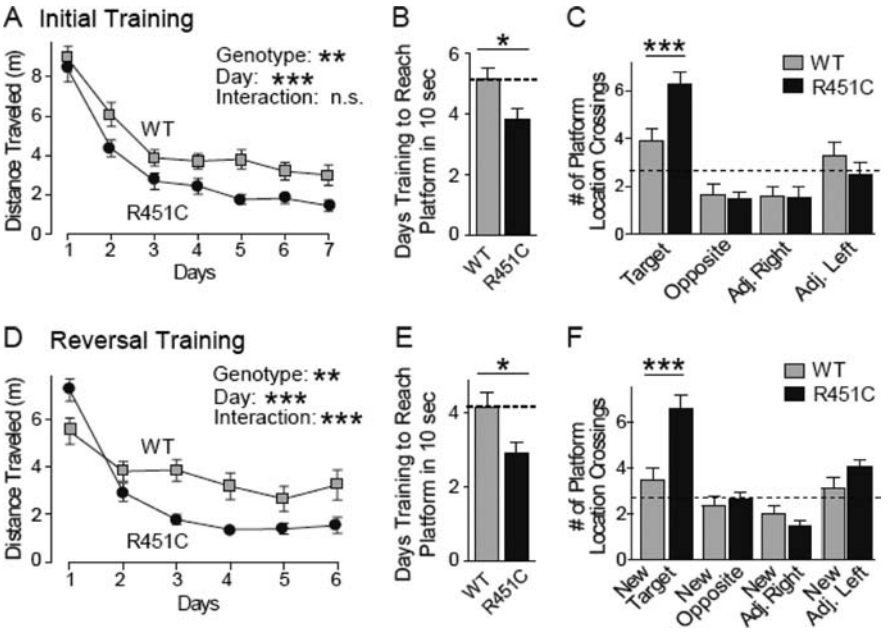

Fig. 4 Neuroligin 3 R451C KI mice exhibit enhanced spatial learning. (**A**) Morris water maze analysis of spatial learning in R451C KI and littermate wild-type control mice during the initial 7 days of training as measured by the distance traveled to reach a submerged platform. (**B**) Number of days of initial training required to reach the submerged platform in an average of 10 s or less. (**C**) Number of crossings over the previous location of the target platform and over corresponding locations in the other three quadrants measured on day 8 after removal of the platform (probe trial). (**D**) Reversal learning experiment in which on day 9 after the probe trial the platform was moved to the opposite quadrant, and the learning of the new location of the platform by the mice was monitored. Learning is measured as distance traveled prior to mounting the newly localized target platform as a function of days of training. (**E**) Number of days of reversal training required to reach the submerged platform in an average of 10 sec or less. (**F**) Probe trial after reversal learning uncovers a large increase in learning abilities of the R451C KI mice. Only statistically significant differences between wild-type and R451C KI mice are identified in the panels ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; in A and D, genotype, main effect of genotype; day, main effect of day of training; interaction, interaction between genotype and day). All data shown are means ± SEMs; $n = 19$ male littermate pairs; see (Tabuchi et al., 2007) for detailed statistical comparisons

retrained (so-called reversal training). Again, R451C KI mice exhibited a significantly enhanced learning curve during (Fig. 4D) and required fewer days of training to learn the location of the platform (Fig. 4E). Twenty-four hours after the final reversal training day, R451C KI mice displayed enhanced spatial memory during the probe trial. R451C KI mice showed a significant preference for the new target quadrant and spent significantly more time in the target quadrant than wild-type littermate control mice (Fig. 4F). Similarly, R451C KI mice crossed the new target location more often than control mice and exhibited a significant preference for the

target location over all other locations, unlike wild-type mice (Fig. 4F), suggesting that they have an increased ability for spatial learning and memory.

The social interaction deficits in NL3 R451C mice are not associated with a broader behavioral syndrome. Locomotor activity, anxiety-like behavior, motor coordination, vision, and swimming ability are unaltered by the NL3 R451C mutation (Tabuchi et al., 2007) (not reproduced here).

Our published data also show that behavioral deficits in NL3 R451C mice are associated with an increase in inhibitory synaptic markers, increased evoked excitatory synaptic transmission, increased numbers of inhibitory synaptic puncta above detection threshold, but no change in the number of symmetric or asymmetric synapses by electron microscopy (Tabuchi et al., 2007) (not reproduced here). This increase in inhibitory synaptic function is consistent with the hypothesis that autism-like behaviors may be due to alterations in excitatory to inhibitory balance.

Interestingly, the NL3 R451C mutant protein has decreased stability as a 90% decrease in NL3 protein is observed with two different NL3 antibodies, but NL3 R451C mRNA levels remain unchanged (Tabuchi et al., 2007). Biochemical and synaptic morphological comparison of the NL3 R451C mice to NL3 KO mice, in which no NL3 is produced, suggests that the NL3 R451C mutation may exert some of its effects on synaptic proteins through a gain-of-function mechanism. Behavioral comparison of NL3 R451C mice to NL3 KO mice will be required to understand if such a gain-of-function plays a role in the behavioral effects of the NL3 mutation.

Following our characterization of NL3 R451C mutants, mice lacking NL4 were also found to exhibit autism-related behavioral abnormalities including social interaction abnormalities and ultrasonic vocalizations (Jamain et al., 2008). The functional consequences of NL4 deletion on synapses have yet to be determined.

Future Questions and Mouse Models

The discovery that synaptic molecules that act to "bridge" the presynaptic terminal with its postsynaptic target are important genetic causes of autism has had an enormous impact on how we think about the pathogenesis of autism. Ultimately, synaptic connections among neurons in the brain connect the outside world to our actions and drive the inner workings of our brain that we call "thoughts." It is perhaps not surprising that so many of the autism genes revealed to date alter synaptic function.

Regarding the neuroligins, several challenges remain. We must try to understand whether alterations in inhibitory synaptic function in the NL3 R451C mice are in any way causally related to their autism-like behaviors. This will require additional information concerning which inhibitory synapses are affected so that specific receptors can be targeted pharmacologically in an attempt to "treat" the autism-related behaviors in this model. Additional work on the effects of NL3 deletion on behavior and synaptic function will be important to determine whether NL3 R451C

mutations truly act via a gain-of-function. NL4 mutations have similar behavioral abnormalities, though the function of NL4 at synapses remains to be determined. Neurexin-1, 2, and 3 triple mutant mice have been published, but neurexin-1 single knockout mice will be examined as a model of neurexin-1 loss-of-function mutations. Finally, shank3, a proposed binding partner of NLs will be an important gene to target for future mouse models of autism.

As new human genetic links to autism are discovered, our ability to understand the pathophysiology of autism through animal models will be exponentially increased. The goals are to recapitulate the genetic cause of autism in mouse models, to determine the relevance of the genetic finding to abnormal behavior in the models, to understand how brain function is altered, and to link abnormal brain function to specific behavioral abnormalities. Once these goals are accomplished, therapeutic approaches will aim to correct the brain function underlying the abnormal behaviors, first in the mouse model, and later in patients with autism. In this manner, human genetic findings will ultimately lead to advanced, specific treatments based on studies of autism animal models.

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The Neurophysical Chemistry of Autism: Postulates from Intelligence Modeling

Peter R. Bergethon

Introduction

A great challenge to progress in the understanding of autism and autistic spectrum disorders (ASD) is the lack of the existence of a unifying theoretical construct that is able to connect the observable behaviors that both characterize and define the autism spectrum disorders to the underlying anatomical and biochemical mechanisms from which those behaviors emerge. Ideally an interdisciplinary paradigm that fosters seamless connection of the behavioral properties of a neurological problem through a mathematically and physically coherent analysis of systems, cells, cell biology, molecules, and then mechanisms at the level of molecular biophysics would be valuable. A research strategy that has promise in this context is the method of intelligence modeling and its supporting theoretical infrastructure. In this chapter this theoretical neuroscience approach will be applied to autism and autistic spectrum disorders with specific consideration of how it might apply fundamental physical rules governing neurological behavior (neurophysics) to help illuminate potential biochemical mechanisms at play in these disorders. The four sections in this chapter will set out the following sequence of ideas:

1. This introduction will briefly outline the importance of systems thinking and modeling. The characteristics of ASD that will guide the application of the intelligence modeling (IM) paradigm will be defined.
2. The IM paradigm will be defined and described along with its array of tools: cognitive dynamics, the cybernetic cycle, the progression of inquiry, and the cycle of pedagogy. Next, the IM paradigm will be illustrated by its application to the case of ambiguous stimuli. This salient problem will be discussed and related to the emergence of autistic behaviors, which will be our concern for the remainder of the essay.

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3. With these tools in hand, further definition of a cognitive dynamics analysis and its insights into the normative flow of information will provide a window into the cognitive dynamics of ASD. The plausible conclusion that autistic behaviors can emerge from non-autistic brains will be briefly explored.
4. Finally the application of the intelligence modeling strategies to propose putative mechanisms that might give rise to the defining behaviors in ASD will be described.

The theoretical methods used in this chapter depend on a familiarity by the reader of the principles and terminology used in systems science (Casti, 1992). From a systems science viewpoint everything is a "system." Everything can therefore be formally described with a systems description:

- A *system* is a set of elements (parts, events, components, and objects both physical and metaphorical) that are connected and that form a complex whole.
- The *properties* of systems are typically emergent from the overall operation of the system.
- *Observables* are properties that are measurable.
- A *description of a system* includes a notation of its
    - *Elements.*
    - *Relationship rules* defining how the elements interact.
    - *Context or background space* in which the elements and rules are found and operate.
    - *State* which is defined by a set of observable properties that are measured together. *Patterns* of observables are typically used to define the state of a system.
    - *Equations of state* that define how the observables are related.

Typically the elements comprising a system are themselves further describable as systems, i.e., they are *subsystems*. System properties are usually distinct from the individual characteristics (or properties) of the subsystem components of the system.

Models are partial descriptions (or abstractions) of systems of interest. A "good" model is an abstraction at an appropriate level of detail that "accurately" represents the reality of the system of interest. The analytical process of this abstraction or "model making" provides both a common language and a uniform process that can ensure communication between entities involved in knowledge processing whether in the brain or among the various disciplines in the practice of science. Staged model making is the central process of the method of modern science. The practice of a cycle of critical analysis, hypothesis creation, and skeptical empiricism to confirm hypotheses and observation is named "the progression of inquiry" (POI) (Bergethon, 2009). Thus, a description of the dynamic of modern science is based on three linked stages of model making. These are the following:

- formation of *descriptive models* that represent observations (of systems of interest);
- generation of *explanatory models* that embed hypothetical linkages of causality (relating how the system of interest works);
- creation of *experimental models* that allow empirical testing of the hypothesized relationships of the explanatory and the epistemological nature of the descriptive models. This empirical testing validates or falsifies the models by comparison of predictions derived from theory (hypotheses) to the measured experimental data.

Many models employ the abstraction that the system under study is static or unchanging over time. Many systems are, in fact, static in the time frame of interest, so this can be an important and reasonable abstraction. However, this abstraction is unreasonable when some aspect of the system as described above changes with time. When changes in system elements, rules of interaction, context, emergent properties, states, or equations of state over time are required for accurate description and explanation of a system under study, dynamic modeling is required. Dynamic modeling explicitly follows changes in the system over time and in many cases a single timescale is inadequate to the task. If necessary, multiple timescales must be incorporated in the model. The mathematical tractability of such models is inversely proportional to the dynamical complexity. Sometimes the dynamical model can be shown to arrive at a dynamic equilibrium and can thus be treated as if it was a static model unchanging over time.

Intelligence modeling is a mixed equilibrium-dynamic modeling technique that incorporates neuroanatomical, chemical, and physiological constraints on a neural system. While the correct choice of constraints is a key to the success of the approach, the technique begins with and depends on the choice of observable behaviors that allow characterization of the neural system into a limited number of definable states. As discussed in Section "The Method of Intelligence Modeling and Cognitive Dynamics", the value of the technique proceeds with the mapping of these behaviorally defined states onto various mathematical and biophysical state spaces, but the initial system description that is almost invariably behavioral is a crucial initial step.

Defining the State of Autism for Input Into the Intelligence Modeling Paradigm

ASD are neurodevelopmental disorders characterized by socially debilitating behaviors with childhood onset. Within the context of the DSM IV, the mental state of "being autistic" or alternatively phrased as "a person having the property of ASD" would be characterized and diagnosed because of exhibiting one of a series of classification patterns whose behavioral observables fall into broad categories of the following:

1. impaired social interaction;
2. impaired communication;
3. manifesting activities, behavior, and interests that are repetitive, restricted, and stereotyped.

The specific observables that the DSM IV measures in order to define the state of ASD are each assigned within these categories (APA, 2000). For example, "impaired social communication" includes deficient regulation of social interaction demonstrated by the loss of the integration of multimodal "systems" information derived from eye contact, facial expression, body posture, and gestures. The observables seen in the impaired communications category include loss of integrated, complex, multimodal, and symbolic aspects of language communication leading to speech that is abbreviated, stereotyped, and unimaginative. The state of "autistic disorder" is defined when observable behaviors from each of the above three categories can be found to exist simultaneously in an individual. The state of "Asperger's disorder" closely maps the "autistic disorder" state except that observables falling in the category of "impaired communication" are necessarily absent. These two states are obviously close in "category space" and have substantial overlap of observable properties, but even this simple analysis raises the question of what underlying mechanisms might be shared or might be exclusive to one or the other to account for the separation between these two systems into meaningfully differentiable states. Examination of the arrangement of the clinical observables into defined states within the pervasive developmental disorders family also suggest that Rett syndrome (RS) and childhood disintegrative disorder (CDD) as mechanistic entities are likely separate from the continuum of "autistic disorder" and "Asperger's disorder." There is a very different dynamic in RS and CDD in that the autistic properties emerge following the establishment of normal behavior. This suggests the ongoing loss or gain of some unifying mechanistic processes whose systems interaction leads to the emergent properties of ASD. Consequently a systems analysis raises the question whether specific behavioral observables of ASD might actually be emergent properties of the nervous system in general. It is intriguing to explore how the overall system of neural machinery might give rise to autistic properties in non-ASD syndromes. In more formal terms, is there an underlying structure to the cybernetic flow of information that gives rise to autistic behaviors in individuals that are clearly not diagnosed with ASD? We will return to this question in Section "The Cognitive Dynamics of Autism."

The Method of Intelligence Modeling and Cognitive Dynamics

A Paradigm for a Unified Theoretical Neuroscience

There is value in the neurosciences, especially in the realms of behavioral and social cognition, in having a set of consistent theoretical paradigms that can be tested in the tradition of the physical sciences. Within the neurosciences, this theoretical tradition

has been most successfully applied at the level of the nerve impulse in the landmark work of Hodgkin and Huxley (1952). Hodgkin and Huxley made great progress in the elucidation of the mechanism(s) underlying the phenomenology of the action potential by careful application of physical and mathematical modeling principles along with judicious use of semiempirical (and therefore experimentally realistic) constraints. The theoretical construct that we are seeking must connect the phenotypic social behaviors of the mind (such as those that can be observed in situations of social interaction, communicative complexity, and cognitive flexibility) to the basic structures, interactions rules, and systemic properties of the biological nervous system and the brain that gives rise to the mind. Intelligence modeling (IM) is a theoretical construct and a research paradigm that applies a progression of linked models to information handling. A subset of the IM approach is *cognitive dynamic theory* (CDT), which is the time-dependent analysis of how manipulation of a stream of data gives rise to a hierarchy of states and subsystems of knowledge used by a neural system such as the brain. IM and CDT are specifically intended to provide a theoretical scientific construct that can be used to characterize and mechanistically understand how the human brain gives rise to complex human behaviors including creative action, knowledge formation/manipulation, and even the development of systems of belief.

IM is a Distinct Program of Computational Neurology

Intelligence modeling is drawn from the intellectual traditions of information theory, systems theory, and cybernetics, three closely linked fields of study. In Section "Introduction," a description of systems theory and the prominent role played by model making in system analysis was introduced. Systems theory is concerned with how the organization of the parts of a system gives rise to the properties of the system independently of the substrate from which the system itself is composed and therefore how a system interaction and emergent behavior can be predicted from general principles (Von Bertalanffy, 1976). Cybernetics is a term introduced by the mathematician Norbert Weiner and is derived from the Greek for "steersman" because it refers to the whole field of control and communications in the animal and machine (Weiner, 1965). Cybernetics is concerned with the flow of information within a system (such as feedback loops) and with the mechanisms governing how information transmitted can act to control the parts or whole of an organism or system generally leading to both homeostasis and intentional action. Information theory (mostly attributed to Shannon, 1948) is concerned with how information is coded and transmitted accurately between a source and a receiver (or between the parts of the system) over transmission channels that are noisy. A key quantity in this treatment is the Shannon entropy (H), a measure of how many bits of information are required for the source to send in order for the receiver to be 100% certain of the state or message of the source. Each bit carries a certain energy cost, and the sending of bits whose state are equiprobable are the most energetically costly to send.

The capacity to send maximal information at minimal *H*-cost is a central concern in information theory, specifically source coding.

It should be emphasized that within a system of interest (whether animal or machine), the sources and receivers characterized by information theory are the elements of a neural system that is itself a cybernetic entity. The brain is a cybernetic entity in as much as the information being processed governs the interaction between the sending and the receiving parts. These parts then act together to provide homeostatic or intentional observable behaviors of the neural system itself. In IM, this flow of information is given canonical form in the "cybernetic cycle" (vide infra). Furthermore, these information flows are in fact models (or abstractions) of some sender state (i.e., the state of the external world or some aspect of the internal milieu), so the formalisms of cybernetic communication system theories are a natural fit for neurological investigations.

The Systems Modeling Tools Used in IM and CDT

Several tools have proved valuable in IM explorations to keep track of the mapping and interaction of abstracted models of neural events. One of these is called the cybernetic cycle and as shown in Table 1 represents the approach of the author and A.E.R. Woodcock, who emphasize the role of context in the process. The cybernetic cycle serves to organize the chain of abstractions, sources and receivers, and interacting cybernetic entities throughout a computational system into a systems/subsystems hierarchy. Each information-carrying element listed on the far left is embedded in its background space (context) and through the interaction rules gives rise to the emergent property (far right column) of that level of the cybernetic cycle. What emerges from one level is the information-carrying element (a new subsystem) of the next level in the hierarchy. Though written in Table 1 as a "bottom-up" linear sequence, it is actually a system of nodes with multiple interconnections. These allow for exchange of information among the nodes so that both top-down and bottom-up interactions are fostered through the "mesh" of chained models. The path through this mesh is naturally described as an information trajectory between nodes of interest. For example, belief and knowledge level information can feedback onto perception processing to alter the cognate level output of a data stream. This type of complex coupling can change processing at the "world view" level.

Table 1 The cybernetic cycle

Interaction in context	→	Stimulus
Stimulus in context	→	Data
Data in context	→	Information
Information in context	→	Perception
Perception in context	→	Cognition
Cognition in context	→	Understanding
Understanding in context	→	Knowledge
Knowledge in context	→	Belief

While the cybernetic cycle is a formalism that focuses on the processing of data streams into complex knowledge structures, the "cycle of pedagogy" represents how these knowledge structures are updated in a learning cycle (Bergethon, 2008). The COP is also expressed in terms of systems analysis with the recognition that three interlinked model systems are interacting during learning. In the COP, learning and ontological growth is dynamically represented by system descriptions of the following:

- the "mechanistic" structure of knowledge production (a nervous system or other computational information processing system) – this is essentially the cybernetic cycle for a given knowledge engine.
- the knowledge structure of a field or disciplinary domain, i.e., facts and their theoretical interactions – content, concepts, and context.
- the path of ontological growth from naive to expert entity within a field of inquiry.

Implicit in this paradigm is the notion that the cybernetic sequence is embedded in the neural, knowledge base, and learned internal models and can be mapped by defining the differential change in mental, disciplinary structural, and expert models.

Intelligence Modeling is a Method of Scientific Model Building

The steps that comprise the IM method are modeling processes that employ a linked systems analysis as described by the progression of inquiry. A descriptive model of a particular behavior is the starting point. A behavior of interest can be "complex" such as the visual perception of a spatially unified scene (absent, for example, in simultagnosia) or more elementary such as the generation of a train of action potentials and their representation of stimulus into a data stream. In IM, all events within the context of the nervous system are treated as systems with emergent properties that can be abstracted, represented, and mapped as a model of the system of interest. Once a description of the behavior is achieved, that description can be "drawn" or represented in graphical or mathematical form. If the system description is small enough in dimension ($n = 1$, 2, or 3 dimensions) it is relatively straightforward to draw the picture and to represent it as a line, curve, plane, or three-dimensional object. When the neural event or stimulus is higher dimension, a simple graphical representation becomes more challenging but mathematical representations of the system of interest remain fruitful lines of investigation. Each element in the system space can be represented as an entity in that space and its relationships to other entities within the system is given by a list of values that can be represented by an n-dimensional vector. Two essential questions always to be considered are as follows:

- How many dimensions (n) are required for the "good" or useful representation of the real behavior of interest?
- What are the properties of the space onto which this n-dimension vector is mapped?

The IM Process

We will now state the process of intelligence modeling. Then its application to a behavior that is salient to the emergence of the ASD state will be examined.

Step 1: Describe, graph, and map the behavior (*POI:* Build Descriptive Model)

- Step 1a: Describe the behavior of interest (systems description).
  - Observable properties are selected to represent state of system.
- Step 1b: Draw a geometrical representation of the description.
  - The pattern of state transitions is mapped, often onto a topological surface. A topological surface (called a manifold) can suggest canonical equations possessing coefficients that can be assigned mechanistic meaning.
- Step 1c: Write the canonical form of the geometry (line, plane, sphere, step) in mathematical form. Ideally, this geometry can be treated as a potential energy surface or some other experimentally accessible variable (see Step 2).

Step 2: Propose plausible mechanisms that meet requirements of the canonical equations or form (*POI:* Propose Explanatory/Causal Model)

- Chose constraints for the system and apply these to limit the solutions to the canonical equations. Constraint conventions are used that limit the choices of how a particular problem can be solved. The problem is "two-way constrained":
- Bottom-up Constraints – this determination is semiempirical and is derived from the actual system under study. This is a point at which IM deviates from general system theory, where the ideal is to find fundamental principles derived solely from the system arrangement and are independent of the actual substrate.
  - Biological constraints, e.g., cells (morphology), anatomy (connectivity), physiology (functional limitations, e.g., conduction velocity)
  - Physiochemical constraints, e.g. – energetics, kinetics, dynamic behavior
  - A dominant and universal constraint applied in IM is the extraordinarily limited energy availability to biological neural systems. This is a frank recognition of the role played by evolutionary forces in shaping the system. For example, the total energy limit for the human brain is 25 W and only 12–13 W is available for computational processes. A key idea is that information fidelity and choice are potentially costly in both energy and time. Biological information systems ultimately occupy states that minimize free energy ($\Delta G_{\min}$) with $G = \mathrm{E} - \mathrm{TS}$. G represents the balance between internal energy cost and physical entropy and can be written with respect to the Shannon entropy as well. Systems with high information fidelity have low physical entropy system states and therefore energy is consumed to achieve these states. Thus, it is natural to find mechanistic solutions that locate both (1) regions of maxima or minima in the potential field (points on the manifold) and (2) paths between

information states in which a minimum of Shannon entropy (and its energy equivalent) and a maximal negative potential energy change result. This is quantified in the measure of "cybernetic action." In general, IM will attempt to write the mechanism in terms that can be measured by state changes in the energy of the system under study.

- Top-down constraints – these are mostly consistent with the general system, cybernetic, and information theory constraints of how a system is assembled and behaves. For example, if a single set of data gives a two-valued observable output, this imposes certain restrictions on mechanistic solutions because not all operations can deliver this solution.

Step 3: Propose testable hypotheses to test the causal/mechanistic model developed in Step 2 (*POI:* make predictions based on experimental observables). Because of the energy constraint in Step 2, experiments that reflect energy utilization (glucose, oxygen, or blood flow in cases of neurovascular coupling) can provide important insight for evaluating theoretical predictions.

Step 4 (*POI*: test in an experimental model): The mechanistic models developed are validated with experimental data. The experiments are inevitably reductionist, but for most "high-order" behaviors, there will be strong systems interactions making reductionist analysis complicated. Therefore, there will be a tension between this tendency to oversimplify the model at the experimental level and the theoretical "goodness" of the model. In order to strengthen the convergence between the observational and mechanistic models, two principles are applied:

- The 2001 Constraint. This whimsical term refers to the computational psychosis suffered by the HAL series computer in Arthur D. Clarke's 2001: A Space Odyssey. A useful computational model, when actually constructed, should be able to demonstrate the same variety of pathologies that the human nervous system suffers. In IM this is considered an essential test of the "goodness of a model."
- The Cognitive Correspondence Principle: Named after the inspiration of Neils Bohr in tying quantum physics to Newtonian physics, this principle recognizes the mathematical hierarchy required in modeling complex systems and need to make simplifying assumptions. It states that each assumption must, in the limit, be found to be a valid truncation of a more precise mathematical description of the overall system.

Step 5: The final step is the replication of the behavior of interest by building a device or machined version of the knowledge mechanism that generates an emergent property equivalent to the original real behavior. Ideally a similar information processing mechanism using realistic constraints is used. This step should be clearly differentiated from the "black box" approach of artificial intelligence workers.

Intelligence Modeling of Ambiguous Figures

As an example of the IM approach we now consider the case of ambiguous figures such as the mother–daughter figure (Fig. 1a) popularized by Boring (Hill, 1915; Boring, 1930) and the face–chalice figure (Fig. 3a) created by E. Rubin (Rubin, 1915).

Step 1: Identify, describe, and map behaviors

The behavioral characteristic of these figures that has fascinated and delighted diverse audiences including computer and neuroscientists as well as children of every age is the sudden jump between two behavioral states. In the case of the mother–daughter picture the figure changes from an old to a young woman. In the perfectly ambiguous Fig. (1a), the figures switch back and forth with a random dynamic gamma distribution. In similar fashion of the Rubin figure changes from a vase to two faces. Thus, two behaviors can be identified from a single data set – this is the property of *duality*. When the change between behavioral states occurs it does so suddenly and dramatically. This is the property of *catastrophic change*. Only one of two states is discoverable, there is not any capacity to discover the partial states that would logically be expected to lead from one image to the other. These undiscoverable states are due to the property of *inaccessibility*. As might be expected and as shown in Fig. 1 and 3B, figures can be altered, so one or the other state is more likely to be seen. The pattern of very slight changes can lead almost imperceptively from one state or the other. This is the property of *divergence*. Finally these alterations can then be arranged so that, for example, by presenting an image favoring

Fig. 1 Boring's mother–daughter figure A: Ambiguous figure; B: Figure biased to young woman; C: Figure biased toward older woman

perception of the young woman (Fig. 1b) or the old woman (Fig. 1c) first, the character seen on presentation of the ambiguous picture (Fig. 1a is the same image as the last image in 1b and 1c) will be strongly influenced by the prior experience. This bias by prior experience is called *hysteresis*. Careful investigation of these figures shows these behaviors.

These observable behaviors cannot be captured on any simple plane or a line. On the contrary, when the five properties listed above are found in a system a specific geometry is predicted. The independent variables that will give rise to dependent behaviors such as these five must be mapped onto a surface or manifold such as that drawn in Fig. 2 and 3b.

This figure is called a cusp catastrophe and is a derivative of a body of mathematics called catastrophe theory (Thom, 1994; Poston and Stewart, 1996). The

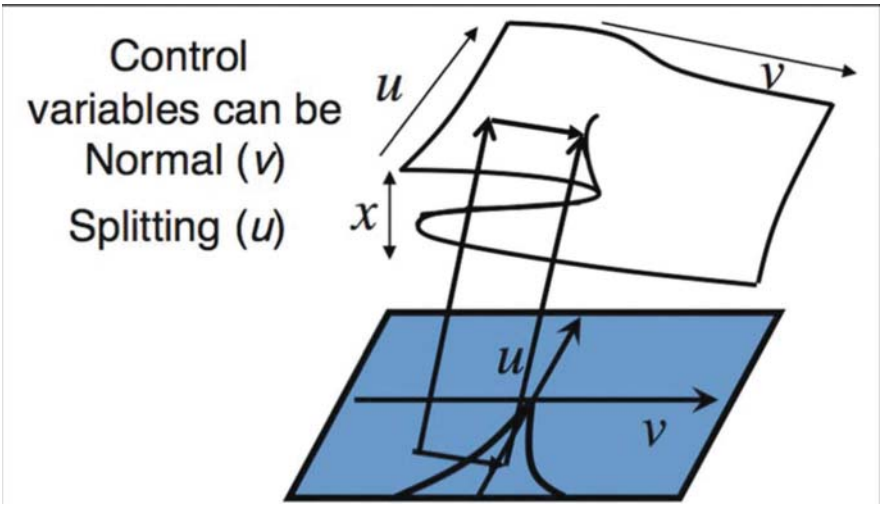

Fig. 2 The geometry of the cusp catastrophe. The lower part of the figure is the control surface in *u* and *v* and its solution is drawn as the manifold (complex surface) above the control plane. The movement across the control surface (from left to right) is projected onto the manifold

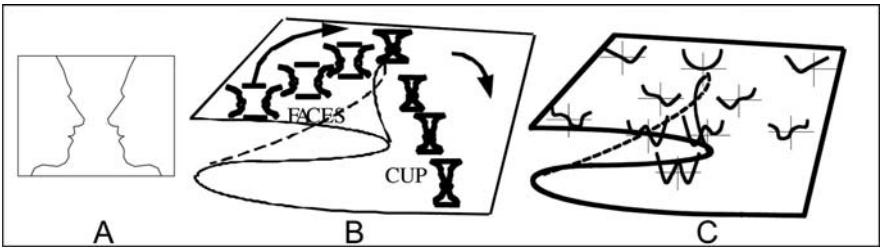

Fig. 3 A: The face – chalice ambiguous figure drawn in B: as a series of altered figures that map onto a cusp catastrophe. The potential energy of each (*u, v*) point as projected onto the manifold

upper folded surface (a manifold) is a phase state diagram that represents the maximal and minimal solutions in x (found by setting the first derivatives of $F(x) = 0$). In Fig. 3, x is the "behavior" that the cup or chalice is perceived and this is represented by the upper or lower sheets. The value of x is determined by two control inputs (u and v) which can be graphed on a two-dimensional control surface seen beneath the manifold in Fig. 2. The control variables are related by the following canonical equation:

$$F(x) = \frac{1}{4}x^4 - \frac{1}{2}vx^2 - ux.$$

.

At this point the complex behaviors of these ambiguous figures have been related to plausible mathematical form. When the behaviors of interest can be described by the behavior of the observable x, a potential mechanism can be found by looking for the identity and system descriptions of the control variables u and v. One of the advantages of this particular treatment is that the manifold is a potential surface of the function of interest. Thus, this mathematical form allows a potential energy cost to be related to a particular behavior. It should be appreciated that other manifolds and equations might be found for other behaviors.

Steps 2–3: (apply constraints and make predictions from mathematical model): There are many constraints that can be applied to this case but as discussed earlier one of the most important is the tight constraint in energy availability to the human brain. Each point on the manifold has an associated potential energy curve. Some of these are illustrated in Fig. 3c. It can be seen that presentation of the ambiguous figure is at the top of the cusp line (Fig. 2b) – in the "flat" zone of the manifold which is at a global energy maximum change compared to a nonambiguous presentation (note that at the top of the fold line the energy minimum is ≥ 0 which is the highest potential energy on the map in 2C). Therefore, the energy utilization in the brain for ambiguous figures would be expected to be higher than the perception of stable figures. This hypothesis can now be tested.

Step 4 (experimental testing): Functional MRI is a tool that by measuring localized blood flow can be used as a proxy for cortical energy utilization. The prediction made above can be tested by examining data obtained by Kleinschmidt et al. (1998) who reported a higher cortical energy utilization when subjects were shown these same figures in the ambiguous form compared to the unambiguous stable images, thus supporting the prediction of the IM methodology. An additional finding in this study was that thalamic activity was reduced during ambiguous stimulation. This discovery suggests that the brain may employ a strategy to suppress information streams causing excessive energy utilization. This supports the idea that brain energy consumption is monitored and homeostatically adjusted to optimize energy utilization. Further support to generalize this hypothesis that ambiguous states in neural processing are energetically expensive can be found in the literature. Studies exploring the process of disambiguating meaning in sentences show increased cortical energy consumption in the ambiguous state (Mason and Just 2007). This raises some interesting mechanistic considerations in the case of the autistic brain. Could

ASD behaviors be a homeostatic response to cognitive challenges that are energetically overwhelming? Possible implications of this idea will be discussed in Section "Mechanistic Considerations in Autism."

Cognitive Dynamics Describes How Brains Assemble and Then Operate on Internal Mental Models

The brain extracts, constructs, and manipulates internal models of the world. This is a well-developed and validated construct with applications in neurology, cognitive neuroscience, education, and the social sciences. There is substantial experimental evidence that the nervous system employs a computational strategy in which sensory data are processed from the level of stimulation to the level of cognition by the process of feature abstraction (Hartline, 1940; Dowling, 1979; Hubel, 1988; Mountcastle and Darian-Smith, 1968). The role of the generated internal models is central to the normal function of the brain and serves as the integral context for all of the actions of the nervous system. These actions include the planning and execution of motor strategies, memory, reflexive learning, declarative (propositional) learning, abstractions in language, graphical, and somatic posture. Neurological syndromes that can demonstrate the abnormalities of the failure to produce or to correctly interact with these internal models are well described (Victor and Ropper, 2001). The central role in IM played by a cybernetic treatment of the flow of information in the brain has been introduced.

In cognitive dynamics, an object or event that will be processed via the chain of the cybernetic cycle is regarded as a system of information elements. A system of stimuli is presented to the hierarchical levels of computational processing represented by the cybernetic cycle. Each level in the cycle abstracts and constructs a model of the original stimulus system generally combining and recombining the stimulus stream with other inputs. The flow of this information represents the stream of "cognitive" knowledge processed within a computational system. Like our discussion of the IM paradigm, the representations of both the information stream and the internal "knowledge state" (hard-wiring) of the cybernetic unit are described by a "knowledge surface" or manifold. The cybernetic cycle treats this series of knowledge surfaces representing both knowledge/learning and also the computational manipulation of an information flow. The use and manipulation of an information stream allows cybernetic control in the system. These connected manifolds are considered as sources and receivers linked via potentially noisy channels and are treated by information theory. There is substantial mathematical complexity in the treatment of this information, and but here we will simply illustrate the process graphically and qualitatively. Then we will consider the cognitive dynamics of the key behaviors that characterize the autistic state.

A completely general and defining function of any information processing system (including the human nervous system) is the capacity to change the state of a processing unit in response to a specific pattern of input(s). Consider the general case where the processing unit is a network of interacting neural elements whose

anatomical and functional connectivity are represented by a manifold that characterizes a target state. The manifold of the target state represents either learned or structurally determined information, so the computational potential or knowledge represented has a geometric form (with each localized point related to an energetic state). The processing unit compares an input stimulus by mapping the feature elements onto the manifold and changing state, represented in its discharge, when the input stimulus matches the target state. If the knowledge surface is noncontoured, there is little distinguishing difference between points on the manifold (the points are equiprobable) and therefore little computational efficiency. This means that more information needs to be mapped or transmitted in order to find a distinctive state representing the knowledge content (a high Shannon entropy case). A manifold with maxima, folds, and minima will be capable of providing more certain knowledge of a new state at lower energy cost (formally this means the Shannon entropy is lower because it takes fewer bits of information to identify the state of the knowledge surface).

Consider how the cybernetic cycle defines the progressive mapping of a data flow representing the image of a dog (Fig. 4). The anatomical arrangement of the processing units in the brain in the following example will be familiar (Fig. 4 – left), but it is the flow of information into and along the manifolds that is most important in CDT. Briefly, the molecules of the dog interact with photons; these photons carry the field of information of the spatial arrangement of the dog to the photoreceptors in the retina where their interaction leads to light stimulus that in the context of the neuroanatomical organization of the retina generates a feature abstracted data steam – a model of the photic field of interaction that was the spatial information of the dog. The data stream moves through the largely deterministic structural context of the lateral geniculate nucleus and primary visual cortex as an information stream that is again a feature abstracted and recombined in higher degrees into percepts, cognates, and knowledge structures that are increasingly knowledge laden. Each level is necessarily more abstracted so that as information models they can be processed with minimum energy. It is worth emphasizing that the manifolds represent the computational sequences in the brain. The most "primitive" or elementary

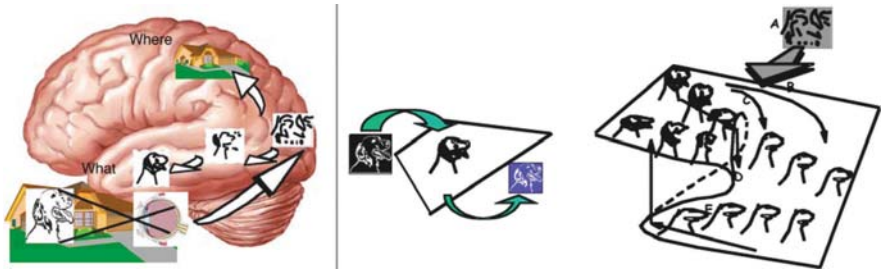

Fig. 4 Left: Cartoon of the image of a dog as the stimulus stream is abstracted and modeled in the brain. Right: Naïve learning requires high point-to-point mapping to define a single object (dog). A folded knowledge surface can categorize and differentiate a class of objects

are largely point-to-point mapping (planar or photographic maps), e.g., information from the optical image focused on the retina (Fig. 4 – middle). These will subsequently become less and less planar at higher levels of processing. The two-dimensional image formed on the retina has an x–y representation (with a z-axis of varying amplitudes) that is then feature abstracted to represent lines, contrasts, and arrangements of feature elements. Thus, the knowledge manifolds above the simplest level of representation (stimulus) will be contoured with maxima, minima, and saddle points – the contours of those manifolds represent the abstracted elements of the internal modeling steps along the cybernetic cycle trajectory. The contouring and folding of the knowledge/computational manifolds is an important part of the process of minimizing Shannon entropy and maximizing the energy efficiency the cybernetic cycle. As shown schematically in Fig. 4, when early learning occurs an information stream is mapped onto a relatively featureless manifold, but one that requires an input to target state mapping that is close to one-to-one, thereby requiring a high Shannon entropy to read out the target state. A richer learning set of stimuli leads to categorical separations arranged so that the mapping of a stimulus set onto the knowledge manifold falls somewhere on the category, providing a categorical computation that is more expert with both less data and simultaneously with clearer separation (using less data) but also a broader grouping (i.e., recognizing sameness even in the face of apparent diversity). In Fig. 4, right, this folded manifold has two broad states or categories (dogs and bears/seals) that will allow the incoming data stream (A) from the top to be compared and categorized but at a higher or more abstract level. Various paths of this data flow are represented in the streams labeled B, C, D, and E. D and E represent a hysteresis path while B and C divergence paths. This codified in the idea of cybernetic trajectory and cybernetic action.

Cybernetic Action and the Principle of Least Cybernetic Action

A key idea in cognitive dynamics analysis is that the flow of information can be envisioned taking a variety of paths across a knowledge manifold. As information flows across the landscape toward stable (and therefore identifiable) behavioral states some paths are favored over others in terms of the overall trajectory of the information stream. A value for each of these paths in a cognitive dynamics treatment is called its "cybernetic action." The cybernetic action relates the energy content of the information stream (the energy associated with the movement of the information is a function of the Shannon entropy and the energy related to the change in information potential on the manifold surface) and the time interval required to traverse the path between states. In general, given the IM energy constraint, the path with least cognitive action is a preferred path since on this path a higher density of information will be transferred or transformed in less time (higher bandwidth) with the least energy cost to the system. The brain would be ontologically driven both in its biology and in its psychological development to process information along

paths of least action. If such paths cannot be found, the resulting higher energy demands by the brain circuitry will have potential negative impacts on the overall brain energy usage. Within the cybernetic cycle, each processing network will seek a path of least cognitive action selected from the interplay of various neurophysiological, neuroanatomical, and neurochemical mechanisms. Thus, the knowledge surface at a given hierarchical level on which paths characterized by varying cognitive action are formed can be altered by biological mechanism (developmental or plasticity). Intrinsic biological (nature) or learning (nurture) can each play varying roles in the attainment of the principle of least cognitive action. If a least action path is not available, the system level interaction of the cybernetic cycle provides the brain with a mechanism to suppress high cognitive action paths at a higher level by altering or ignoring details of the information stream.

The process of seeking out a trajectory across a knowledge manifold can be imagined as analogous to the process of diffusion in a potential field and in brief involves steps or jumps along the manifold (named the diffusion model of intelligent action). The length of the step or jump, the frequency of the step, the landing or binding of the step to the potential surface, the likelihood that a step is random or in a preferred direction depending both prior history or potential field, and the amount of information being moved across the surface are all defined properties of the process of forming cybernetic action trajectories. Thus, the cognitive trajectory can be mapped as a path of a packet of information over a potential energy landscape.

A final feature of the principle of least cybernetic action is that as the cybernetic cycle is traversed through sequential models, the dimension or number of observables necessary to represent the model grows either very slowly or is constant. This requires careful abstraction of a model so that while the "goodness" of the model remains high, the cost of representing the model is low. From an IM energy constraint perspective, this is consistent with the needs of evolutionary neurobiology. It can be shown in a variety of mathematical formats that if the dimension (size) of a data field is not constrained, the demand on the size of the computational machinery grows exponentially. As an example, the computational costs to perform operations on a n-dimensional matrix is $\propto n^3$. Thus, as the size of the matrix needed to represent a data set such as the pixels used to form a dog's figure grows, computational costs and time for each cybernetic step grows exponentially. Cognitive dynamics looks at how sequences of model making modules can be arranged to process the increased content while successfully meeting the time and energy constraints on the costs of computation.

A Cognitive Dynamics Treatment of Dogginess and Bearishness – Complex Knowledge Interactions Map to Complex or Interacting Surfaces

Figure 5 provides a schematic representation of how information flow can be sequentially abstracted and then mapped onto a series of manifolds that tend to reduce the dimension (and therefore limit the cybernetic cost). The general idea

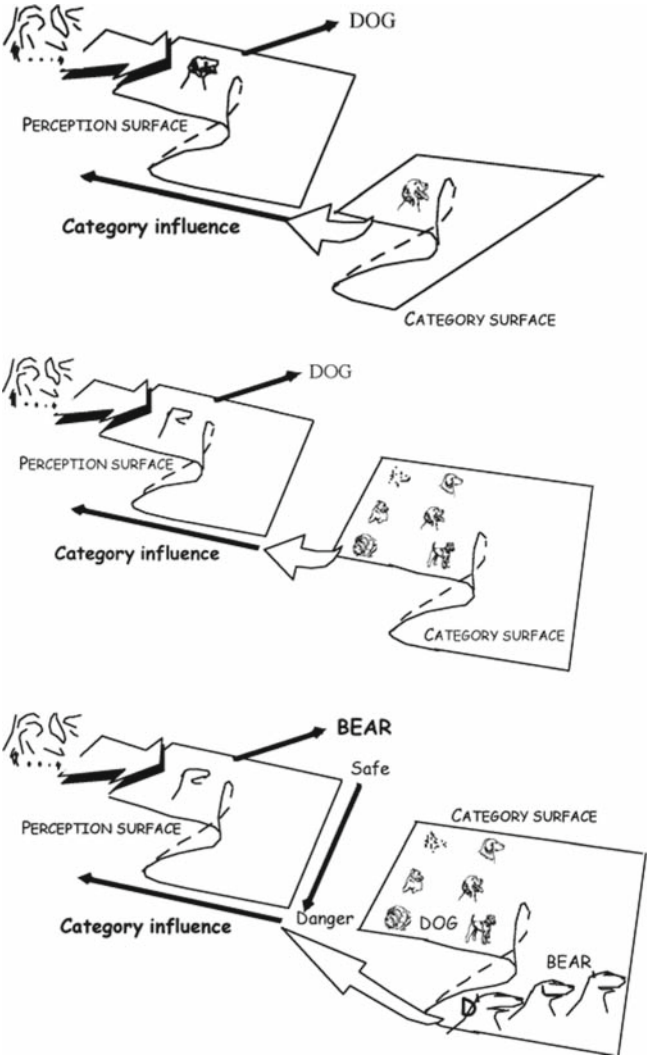

Fig. 5 Hierarchy of knowledge manifold interactions as described in text. Top: Minimal input from naïve category surface forces "complete" modeling at level of perception – surface to identify "dog". Middle: More complex category surface (more experienced) allows less perceptual modeling (more abstract) to reach the state of "dog". Bottom: Category influence in the content of a safe or dangerous environment influences minimal data stream to generate two different cognates, either a bear (dangerous) or a dog (safe)

of this type of cybernetic processing is that as the models represented by information streams become more sophisticated they become more formalized and more abstract. This serves to keep the dimension of the model small, thus allowing efficient processing but at the cost of an ever more abstract model. In Fig. 5, the top figure shows how a knowledge surface with little "expertise" or minimal knowledge of the category must force a much more detailed exploration and extraction

of data from the environment at high Shannon entropy and higher energy cost compared to the next figure. In the middle figure, possession and access to categorization knowledge (expertise) substantially reduces the Shannon entropy and energy cost to determine the state of a cognitive event by biasing the expected output of the source (the environment or more precisely, the perception circuits). In the bottom figure, an example of how a limbic state of anxiety from a danger/safety axis (u-axis) will influence the knowledge state of the overall system by interacting with the perception surface to a novel stimulus. In this case the touch of a cold nose, in the dark, in the safety of your home or cave is enough data to "know" that it is your dog, but if you are in the woods, which is a dangerous place, the same cold nose sensation will generate "bear," not dog, knowledge. Highly complex cognitive tasks like those involved in solving systems of interactive language, social interaction, and the handling of complex systems holistically would require this ability to abstract to remain intact. For example, the power of a metaphorical expression in language captures complex information without the detail. If the detail must be replaced, a much larger data set is required which lists those details. Working through that list in an exhaustive fashion has a relatively high computational cost in terms of both time and energy utilization. It is evident that the "state of ASD" is characterized by types of cognitive tasks that demand very high computational resources.

The Cognitive Dynamics of Autism

In Section "Introduction," the behavioral patterns that define the state of autism were stated. From an IM and CDT perspective all of the impairments seen in ASD are unified. At the systems level all share essentially the identical failing. This is particularly valuable because such a general systems failure implies a common mechanism that can be searched for at the fundamental chemical or functional level. The simplest way to unify all ASD behaviors is to recognize them as variations on the theme of simultagnosia or the failure seen when the elements of a system of dimensional relationship are not recognized as part of a unified whole. In general systems terms, simultagnosia is the failure to see that the whole is formed by the sum of the parts from which it is made. In neurology, simultagnosia is part of a posterior parietal syndrome and is formally diagnosed when a complete system formed from visually oriented elements cannot be perceived and treated as a complete spatially related whole. However, it is an example of a larger group of canonical computational processes that relate the multidimensional orientation of elements to a common origin originating at the involved individual. This means computationally that each object can be related to one another and also to the egocentric space of the individual. The unification of spatial objects into a whole is an exemplar of the more general problem in the computation of a system – the elements, interaction rules/relationships, and the background context or dimension into which the model of the whole is embedded. At a systems level, it is impossible to regard the parts of a system in a unified or holistic manner – the same general failure characterizing the behaviors

seen in ASD. The capacity for joint attention and for integrating gestures, sound and language is computationally the same process as integrating a spatial scene. Thus, failures in joint attention, integration of modalities in language and difficulty treating systems holistically rather than focusing on parts, are all equivalent in a cognitive dynamics scheme. We will show the energetic and cognitive action costs of this failure below.

All of the behaviors that characterize ASD can be characterized in CDT as having a knowledge landscape that is a relatively flat low-dimensional surface compared to the manifolds of cognitively robust normative behaviors. The point of origin for a trajectory of cognitive action on a flattened surface will be relatively less favored in terms of cybernetic action because the knowledge jump away from that point will be likely to return to the same point with little binding interaction at the landing point (no significant potential energy difference on the manifold) that might otherwise tie various informational elements together. The elements on this surface will be energetically isolated and assembling the parts into a holistic system will take more time and more jump energy, and because they are relatively unbounded. Complex exploration on such a surface will have a cognitive action of greater magnitude compared with a more complex, highly folded manifold. Complex self-referential processes that use multimodal information exchange such as combining an affirmative sound with an affirmative gesture or that would display one internal state and then check to see if that internal state is acknowledged have at minimum two requirements. First, the knowledge manifold must have focal contours that localize the cognate of interest, and second these cognate contours must be co-localized so that they can have a high probability of being associated. This manifold structure is qualitatively the same (or more properly, homeomorphic) for actions that require multiple nonverbal behaviors to be coordinated such as simultaneous use of eye contact, facial expression, body postures, and gesture. The formation of co-localized and accessible knowledge surfaces that can be interrogated, and can influence the "self-state," is homeomorphic for developing actions that characterize "theory of mind." Such manifolds would be seen in social and emotional reciprocity as well as the sharing of interests and pleasure with others. The complex social information that is necessary to interact with the faces and postures of other people is overwhelming if this information is represented on a flat surface that needs extensive, essentially new exploration with each passing event. Both the time and the ability to recognize and remember a face will be impacted by this type of manifold making recognition slow and memory of what has already been seen poor. This is precisely the description given of the experience a person with ASD experiences when looking at faces in a social situation (Grandin, 1996). Normal social and facial/body posture information is highly dependent on prior information, i.e., complex social information is computationally tractable only if bias and hysteresis as discussed earlier is built into the knowledge manifold.

Manifolds capable of the representation and computation of the associated meaning of face and gesture change would be expected to be of the same form. Such systems of information are all likely to be highly ambiguous. An essential task of these manifolds is to disambiguate the many possibilities that social and emotional

contexts provide. These problems will have solutions that are complex in the space of least cybernetic action and the successful search will require bias and hysteresis as well as catastrophe if the constraints of minimal cognitive action are to be met. Such a manifold will be required for any highly formalized, metaphorically rich, or complex task that requires many connected sequential choices. This manifold is homeomorphic for computing formalisms, imaginary play, and language. All of these are systems built from sets of elements that are arranged according to specific rules that lead to virtually infinite possible outcomes. For example, take the case of language. The elements of language, words, are arranged in two classes, content words (e.g., places, objects, actions – the class is infinite in size) and relational words (e.g., on, into, behind – this class is actually quite small). The arrangement of the words according to the rules of syntax imparts meaning to the system of words that form sentences. Varying the arrangement of just 20 words in the English language can give rise to upward of 10 million trillion grammatically meaningful sentences (Dronkers et al., 2000). Language formation, like imaginary play, requires a series of choices in which the prior arranged element continuously creates opportunities in a possibility space that must be correctly chosen both for communication to occur from sender to receiver or vice versa. In this situation the information bits are not equiprobable and the Shannon entropy is lowered. A flattened manifold has a high Shannon entropy and a substantial combinatoric cost to find a trajectory of least cognitive action. Thus, a cognitive dynamic treatment of autistic behaviors predicts a unifying mechanism at least at the computational level. The dynamics of the autistic behaviors can result from a failure to form the high-dimensional surfaces. According to this analysis, the low dimension, flattened manifolds that are predicted in patients with ASD will not respond or even perceive ambiguous stimuli in the same fashion as normal subjects since they do not have access to the fold and cusp lines of the manifold. A recent study of the response to ambiguous figures in people with Asperger's syndrome have confirmed this prediction (Stoesz, 2008).

Does Migraine Provide Insight into ASD?

Imagine that an autistic brain that is restricted to these flat low-dimensional knowledge manifolds finds itself having to solve the problems that require very large computational resources. The simplest solution to reducing computational demand would be to remove the demand. An autistic mind when presented with computationally treacherous territory will avoid the interaction or if denied the opportunity to remove the irritant would be expected to react with anxiety to the intractable problem with which it is faced. Some of the behaviors associated with autism are not unique to it but are certainly homeomorphic displays seen when a brain is exposed to unresolvable irritants. For example, the high energy cost of social interactions during migraine headache is limited by the profound withdrawal behaviors of the migraneur and pain patient including restrictions in

communication, minimalist language interaction, withdrawal from initiating or continuing conversations, and even rubbing, flapping, rocking, or repetitive motions (Lovati et al., 2008; Sullivan et al., 2000). Many similar patterns of action are seen in OCD, chronic pain, depression, and drug-dependent behaviors (McDougle et al., 1995; Roffman and Raskin, 1997.) – clearly indicating that the behaviors are a far more general output pattern of brains. The hypothesis that these "avoidance" behaviors may be directly caused as a result of circuits warning of energy and information depletion is derivative of our analysis but remains to be proven directly.

Mechanistic Considerations in Autism

The cognitive dynamics analysis of autistic behaviors can be unified with the view that the brain exhibiting autistic tendencies struggles to achieve energy homeostasis in its information processing dynamic. Two alternative mathematical formulations both leading to the same general conclusion have been suggested by our treatment in the previous section.

In the case of a matrix solution with large dimension, the computational cost can become too large to be sustained and solutions that are useful are obtained by either reducing the dimension (or size) of the matrix or by maximizing the sparseness of the matrix (the number of cells with a value of zero). This is a mathematical statement of making a model as simple as possible, bounded on one end by the model being computationally tractable and on the other end by being a "good" model. Regardless, information must be destroyed in the real process and the ideal neural machine will destroy the greatest amount of information that has the least utility. As we will see, there are neurobiological correlates to this matrix solution, the most obvious of which is the "pruning" of dendritic branching seen in maturing and learning neural networks.

The second mathematical form that has been described is the formation of the catastrophe manifolds of which the cusp catastrophe has been illustrated in Section "The Method of Intelligence Modeling and Cognitive Dynamics." The cusp catastrophe has two control variables, a splitting variable and a normal variable that govern where on the manifold the output (x) function will be found (Fig. 2). When the splitting variable is > 0 the manifold is essentially flat and planar (as well as relatively high in internal energy). When the splitting variable is <0 the output x will be one of two states governed by hysteresis and bias but will be well suited for producing expert type output. This analysis suggests that autistic behaviors are associated with high-value splitting variables while more holistic systems analysis and paths of least cognitive action will be associated with lower valued splitting variables. Figure 5 showed how the informational content represented by several cusp catastrophe manifolds can interact by combining the output (x) of one manifold as a control variable to another manifold. This type of complex cybernetic interaction is an important consideration in the cybernetic cycle. Interactions can

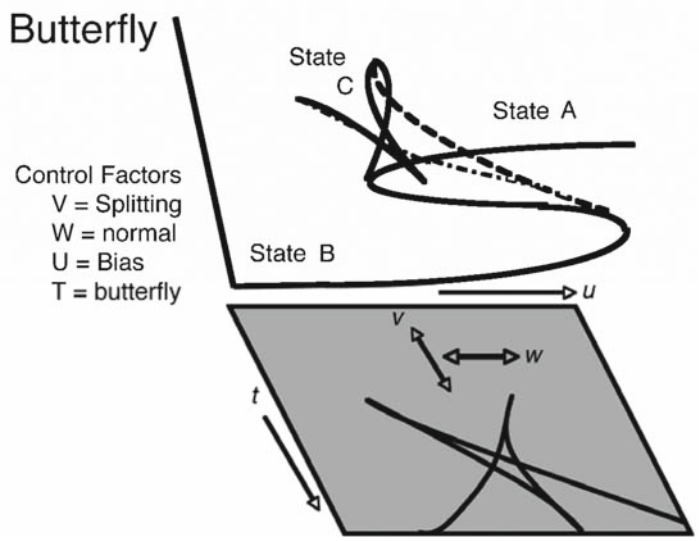

Fig. 6 The butterfly catastrophe generates three states of the output variable *x* controlled by four control factors

also be seen in manifolds of higher dimension that contain more complex folds than the cusp catastrophe. For example, another cuspoid catastrophe, named the butterfly, has four control variables and a single output (*x*) variable. The butterfly is also a useful manifold for describing behaviors seen in neural systems. The portion of this four-dimensional structure that is accessed, and, on which a computation is made depends on the values of these four control variables (Fig. 6). Whether through linked or higher dimension manifolds the regions of these surfaces that give rise to autistic behaviors will be those that are the relatively flat, resulting from control inputs causing high-valued splitting variables.

What are the plausible anatomical and chemical mechanisms that could lead to knowledge surface behaviors in these "autistic" regions versus more adaptable, flexible, and holistic system-savy behavior? The potential mechanisms are unified in cases in which the brain enters a state of excess and unsustainable energy demand. A metaphor that captures the final pathway to ASD-type behaviors is that there is a monitor in the brain that acts like a cognitive circuit breaker: When the computational demand requires an information flow (like current) in excess of the capacity of circuitry in the system, the circuit breakers open or at least shunt the information flow so that the overall integrity of the brain circuits is preserved. The net effect of the rerouted information flow on one hand produces the output behaviors seen in ASD. The preservation of cognitive function until the emergence of anxiety and social withdrawal occurs is consistent with this mechanism. In some cases this compensation can be a conditioning stimulus to the affected brain. It would be expected that the compensation would cause biological changes such as cell death or receptor modification. These changes will alter the cybernetic structure of the brain over the

long term. In this view, a measurable portion of autistic behaviors would in fact be learned responses and, to a degree, homeostatically appropriate to the underlying biological failures of the nervous system.

Which neurological structures might be likely candidates for a cybernetic action monitoring station and circuit breaking role? Prima facie treatment would argue for structures that are highly connected to the entire neuroaxis, thus able to access neural information on a continuing basis. Furthermore, if cybernetic action homeostasis is an evolutionary requirement for survival and species success, there should be a phylogenetic preservation and probable evolution of these structures over the course of neurological time. Three neural systems are natural contenders for this cybernetic action monitoring system: the thalamus, the amygdala, the cerebellum. Both the thalamus and the cerebellum are in the position of receiving virtually universal primary input from both sensory and motor systems and have extensive reciprocal circuits designed to compare both input and output signals. In a true sense these are input monitoring devices weighted to a data stream that enters the neuroaxis and could easily serve as the forward- and back-propagation circuits required in the cybernetic sequence. In contrast the amygdala is a monitoring station that sits at the final position for monitoring cybernetic action weighted toward the final output mode. Neuroanatomically, the amygdala samples input from the entire neuroaxis and has outputs to essential homeostatic and control circuitry through the central nucleus including widespread modulatory (norepinephrine, dopamine, serotonin, and 5HT) and hypothalamic control projections. The identification of these structures as likely cybernetic action monitoring sites is not meant to be exclusive and the extensive interconnection between them may well suggest more integrated and broader monitoring and response system for neural energetics on a very tightly linked neuroenergetic timescale. The exquisite dynamic of neurovascular coupling being discovered in the brain and nervous system favors such a relationship. Interestingly there is evidence for abnormal anatomy or physiology in each of these structures in ASD (Tsatsanis et al., 2003; Muller et al., 1998; Chugani et al., 1999; Young et al., 1999).

It can be hypothesized that if cybernetic action homeostasis underlies the emergence of ASD behaviors, either the "circuit breakers" themselves are defective or that the underlying cognitive dynamic circuitry is faulty. Failing to find paths of least cybernetic action forces excessive energetic demands on the neural system with the appropriate "circuit breaker" action, swinging into action, thus protecting the overall homeostasis, but with the emergence of the ASD behavior. At the behavioral level, the alternative underlying mechanisms would be indistinguishable. We will proceed with the idea that there is nothing special or unique about the actual behaviors associated with ASD since all of them either alone or in varied constellations can be found in a variety of other disorders. For example, the "autistic" properties seen in migraine such as sensory overload and irritability likely represent circuit breaker-triggered pathways.

In conclusion, three broad categories of mechanism that might be expected to lead to ASD behaviors:

1. The putative circuit breaker system is defective while the cognitive circuits are normal. In this scenario, the abnormal circuit breakers inappropriately trigger energy-avoidant behaviors when the naturally computationally intensive tasks that involve language processing, social interaction, flexibility, and metaphorical thinking are invoked. The inappropriately invoked energy reduction behaviors are likely to induce plasticity changes that permanently alter these circuits and are seen as an ontological change in the individual. The findings described by Bauman and Kemper (2005) of shifts from large well-formed cells seen in preadolescent autistic brains to small, pale cells would be consistent with changes that occur as a result of attempts to modify the homeostatic inconsistencies over time.

   There is substantial neuropathological evidence to support abnormalities in the structure and organization of the cerebellar circuits at the anatomical level and also at the biochemical level which might support the role of the cerebellum as an essential part of the "homeostatic circuit breaker." While from a computational standpoint the amygdala or thalamus would be plausible sites for such a monitoring system, there is less evidence to date for neuropathological abnormalities involving these structures. The suggestion by Bauman and Kemper(2005) that the cerebellar derangements occur early in prenatal development is consistent with the idea that an abnormal "circuit breaker" system capable of measuring homeostatic energetics might be functioning normally until more complex computational processes stress the cerebellar circuitry and bring it into a failure mode. Reports implicate alterations in inhibitory GABA receptors in the brains of patient's with autism (Blatt et al., 2001; Guptill et al., 2007) and reductions in the biosynthesis of GABA because of reduced glutamic acid decarboxylase in the cerebellum (Fatemi et al., 2001; Yip et al., 2007, 2009). The loss of inhibitory inputs is likely to increase the excitatory glutamatergic output in the cerebellum in autistic brain tissue. It is plausible that the resulting increased excitatory output from projections from the deep cerebellar nuclei via the thalamus provides a signal that then projects to the cortex where it elicits the avoidance behaviors seen in ASD.
2. The second group of mechanisms that would lead to ASD behaviors would occur as an appropriate response by the homeostatic energy monitoring circuitry when the computational processes cannot form knowledge manifolds or find paths of least cognitive action efficiently. The circuit breaker systems are intact and respond appropriately to computationally defective circuits that consume excess energy. Here one of two broad mechanisms will fail.

   In the first, the incapacity to form an appropriately folded high-dimensional knowledge manifold(s) capable of efficient processing of the computationally complex systems tasks leads to severe energy demands. The integration of information into the knowledge manifold would be based on the normal biochemical mechanisms for learning and memory integration within the brain. The detailed complexity of a manifold capable of categorization and systems processing will depend on the simultaneous integration of control variables. Our simplest treatments have required at least two simultaneous control variables, but in fact it is far more plausible that at least four control variables would interplay in the

formation of complex knowledge manifolds. Changes in the control variables can lead to the energetically inefficient manifold formation. Thus, the neurobiological mechanisms that might be invoked in this pathological process would be at the level of long-term potentiation and depression within synapses, loss of inhibitory influence leading to over-excitation of synaptic circuits, and the failure to appropriately prune and promote maturing of dendritic organization. All of these mechanisms have been variously described in the literature but none have been generally found in studies up to this point (Pickett and London, 2005; Bauman and Kemper, 2005; Lam et al., 2006). However, given the often normal capacity for learning and high function in many subjects with ASD, these deficiencies are likely not universal and are only variably involved as a central mechanism in ASD.

Even if the development and formation of knowledge manifolds is largely intact (as would be expected in syndromes such as Asperger's) an alternative mechanism that will lead to computational inefficiency can be proposed. The computational process captured in the diffusion model of intelligence requires both patterning of jumps (jump length, jump frequency, jump vector, or direction) and a region of exploration. The process of searching a knowledge surface therefore requires a system with many of the properties found in the cerebellar–basal ganglia circuitry to meter the jump pattern. The region of exploration can be viewed as being set by the splitting variable such that when the splitting variable (u) is large (>0) the manifold will be essentially flat and energetically costly to explore and resolve. Smaller magnitudes of u will lead to processing within the more computationally efficient folded zones of the manifold. The magnitude of the splitting variable could be reflected in the neuromodulatory effects of broadly projected fiber systems such as serotonergic, dopaminergic, or adrenergic neurons. The dynamic of neuromodulation is such that the relatively longtime effect of these projection systems provides an adequate time frame for exploration of the knowledge region. This coordination of jump pattern and regional access is necessary for the determination of the path of least cybernetic action and combines a dynamical coordination of oscillatory activity which is a much faster dynamic with the "permissive" timing of the neuromodulation. Without the correct balance even in the face of the appropriate formation of complex manifolds the portion of the knowledge manifold that can be accessed and explored is the flattened region that is energetically expensive and homeostatically unfavored. This failure of proper "cognitive attention" therefore will lead to ASD behaviors.

3. Finally, a third wholly bioenergetic alternative mechanism can be proposed even in the face of normal circuit breaker and normal computational machinery. If the cellular systems (either neural or glial support or both) are not competent to generate adequate energy reserves on demand, the presence of high computational loads will still trigger an appropriate protective response from the circuit breakers because of a general cybernetic inability to deliver neural energy demands when needed. Biochemical defects at this level could be postulated at the level of the mitochondria, within the redox electron transfer systems at the cellular

level, in neurovascular coupling, and in the maintenance of the metabolic milieu and signaling systems of the glial, neural, and vascular compartments within the brain.

In summary, the application of the intelligence modeling approach leads to the proposal of several mechanisms, all unified by the central constraint of the limited availability of energy in biological systems. Though the mechanisms have been proposed as if they are mutually exclusive, this is obviously not a requirement and is even unlikely. Autism spectrum disorder and the behaviors that define its state are multifactorial and the combination of theoretical mechanisms suggested in this chapter, even when proven as operational in the disease process, are likely to interact in a complex biology to give rise to the wide range of nosologic disease that represents the spectrum of this disorder.

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Pharmacological Treatment of Autism

Terrell T. Gibbs

Introduction

Although the etiology of autism is not understood, the defining or "core" symptoms of autistic disorder are considered to be impaired social interaction, impaired verbal and nonverbal communication, and restrictive, repetitive patterns of behavior. In addition, most patients with a primary diagnosis of autism exhibit other neurological or psychiatric symptoms, which may include seizures, sleep disorders, anxiety, panic attacks, attention deficit/hyperactivity, self-injury, and cognitive impairment (Simonoff et al., 2008). It is not known to what extent these comorbidities reflect the primary pathology of autism and to what extent they represent unrelated vulnerabilities that are exacerbated by the impaired social interaction and communication that is characteristic of the disorder.

For the most part, the core symptoms of autism have so far proved to be largely refractory to pharmacological modification, and therapeutic strategies center upon educational and behavioral interventions (Bodfish, 2004; Myers et al., 2007; Rogers and Vismara, 2008). Nevertheless, most autistic patients receive some form of pharmacotherapy, most of it (with the exception of risperidone) off-label (Oswald and Sonenklar, 2007). In the absence of any real insight into the neurochemical basis of autism, pharmacotherapy is essentially empirical. The drug regimen for a particular patient is typically arrived at by trial and error, based on subjective evaluation of therapeutic efficacy by parents, physicians, and therapists. Most commonly, the therapeutic goal of pharmacotherapy is to suppress troublesome behaviors that are seen as interfering with education and behavioral therapy, but evidence of the benefits of this strategy is limited.

Although there is a great unmet need for development of improved pharmacotherapy for autism, as well as for improved evaluation of existing pharmacotherapy, this has proved problematic for a variety of reasons. Symptoms of autism

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manifest in early childhood, and a developing child presents a moving target with respect to evaluation of severity of symptoms. In the absence of an objective test for autism, evaluation of symptom severity is necessarily based on behavior checklists (reviewed by Scahill, 2005) that are to some extent subjective and thus vulnerable to expectancy bias. Parental consent for a placebo-controlled study of children may be difficult to obtain. Most autistic patients are already receiving some form of pharmacotherapy, which potentially could confound or mask the effects of the study drug. Ideally, participants should be withdrawn from their existing medications, but this is problematic if the existing treatment regimen is perceived as effective. On the other hand, exclusion of patients who appear to be responding to ongoing treatment with another medication could potentially bias the study population by eliminating a subgroup of patients who might be more likely to respond to pharmacotherapy.

As a result of these difficulties, only a few of the medications commonly employed for treatment of autism spectrum disorders (ASD) have been subjected to randomized, double-blind, placebo-controlled studies. Most published studies are uncontrolled, open-label studies of small numbers of patients. Because of the high incidence of response to placebo frequently observed in controlled studies of autism therapies (Aman et al., 2005; Sandler et al., 1999; Belsito et al., 2001; Sandler, 2005), open-label studies are frequently misleading and are of little value for assessing treatment effectiveness, although they can be useful for identifying serious adverse effects.

Secretin: A Cautionary Tale

The difficulties associated with evaluation of autism pharmacotherapies are well illustrated by the example of secretin. Secretin is a 27 amino acid polypeptide that has been shown to regulate duodenal secretion of bicarbonate. In 1998, a case report appeared in which three children who received a single intravenous injection of secretin during diagnostic endoscopy for chronic diarrhea were described as exhibiting dramatic improvement in behavioral symptoms of autism (Horvath et al., 1998). This account was widely reported in the popular press, leading to widespread public interest in secretin therapy for autism. It is estimated that over 2500 children received secretin for treatment of autism, with off-label use of the compound so widespread that the manufacturer had difficulty meeting the demand (Sandler, 2005). Unfortunately, multiple controlled studies have consistently failed to identify any appreciable benefit of secretin over placebo after either single or multiple doses in any ASD population (Esch and Carr, 2004; Sturmey, 2005). As is frequently the case in clinical trials of autism treatments, a high rate of response to placebo was noted in these studies (Sandler, 2005), emphasizing the importance of double-blind, placebo-controlled experimental designs for evaluating the efficacy of ASD therapeutics.

Antidepressants

Antidepressants constitute the most widely prescribed class of psychoactive drugs used for treatment of ASD in children (Oswald and Sonenklar, 2007). In particular, selective serotonin reuptake inhibitors (SSRIs) account for the greatest global market share of medications for ASD (King et al., 2009). The most commonly prescribed antidepressants for treatment of children with autistic disorder in 2002 (in descending order) were paroxetine, sertraline, fluoxetine, citalopram, and fluvoxamine (Oswald and Sonenklar, 2007). A frequent goal of antidepressant treatment is to reduce repetitive behaviors associated with ASD by analogy with the effectiveness of antidepressants in treating perseverative behaviors characteristic of obsessive–compulsive disorder (OCD). In addition, there is some evidence that autism is associated with abnormalities in serotonin neurochemistry (Lam et al., 2006), raising the hope that normalizing serotonin-mediated neurotransmission could lead to improvement in core symptoms of autism (Bethea and Sikich, 2007).

In an early placebo-controlled study, clomipramine, a tricyclic antidepressant with moderate selectivity for inhibiting serotonin reuptake relative to norepinephrine reuptake, was tested against both placebo and the less selective tricyclic antidepressant desipramine. Following a 2-week single-blind placebo trial designed to screen out placebo responders, 12 autistic subjects, with ages ranging from 6 to 23 years, underwent a randomized, double-blind crossover trial of clomipramine and placebo. Dosage was initiated at 25 mg/day and individually titrated over 2–3 weeks, with a maximum dose of 5 mg/kg/day or 250 mg/day. Clomipramine was significantly superior to placebo in reducing abnormal behaviors as rated by the autism relevant subscale of the Children's Psychiatric Rating Scale (CPRS) and obsessive–compulsive behavior as rated on a modified version of the Comprehensive Psychopathological Rating Scale. A parallel crossover trial in a second group of 12 subjects similarly found clomipramine to be superior to the tricyclic antidepressant desipramine, which is moderately selective for inhibition of the norepinephrine transporter. Although adverse effects were not statistically significant, one subject with no prior history of seizures was dropped from the study after experiencing a grand mal seizure while taking clomipramine (Gordon et al., 1993). Seizures are a known adverse effect of tricyclic antidepressants in general and clomipramine in particular. This is of particular concern with respect to autism, which is frequently associated with epilepsy (Simonoff et al., 2008). In addition, dosage had to be reduced in three patients due to adverse cardiovascular effects (prolonged QT interval or tachycardia) (Gordon et al., 1993).

A double-blind crossover trial in which clomipramine was directly compared to haloperidol and placebo in 36 autistic adolescents and adults failed to demonstrate any advantages of clomipramine over haloperidol with respect to Aberrant Behavior Checklist (ABC) measures of hyperactivity and irritability. Although no serious adverse effects were reported, tolerability of clomipramine was significantly worse than haloperidol, with over half of the subjects being unable to complete the clomipramine phase of the study due to adverse effects and/or lack of efficacy. An open-label study also reported a high frequency of adverse effects (Sanchez et al.,

1996). More recent studies have focused on the SSRI class of antidepressants, which do not increase the risk of seizures and carry reduced risk of cardiovascular and autonomic adverse effects.

An early 12-week double-blind, parallel-group study examined the SSRI fluvoxamine vs. placebo in 30 autistic adults. Treatment was initiated at 50 mg/day and individually titrated over the first 3 weeks, with a maximum dosage of 300 mg/day. Fluvoxamine was significantly superior to placebo with respect to overall improvement on the Clinical Global Impressions (CGI) scale, as well as reduction of repetitive thoughts and behavior on the Yale–Brown Obsessive–Compulsive Scale (Y-BOCS), improvement on the maladaptive behavior subscale of the Vineland Adaptive Behavior Scales, decreased aggressive behavior as measured by the Brown Aggression Scale, and improvement on the language subscale of the Ritvo–Freeman Real Life Rating Scale (RF-RLRS). Approximately half of the patients in the fluoxetine treatment group showed a positive response to treatment, compared with none in the placebo group. Aside from transient sedation and nausea in a few patients, fluvoxamine was well tolerated (McDougle et al., 1996). In contrast, a similar placebo-controlled study of 34 children and adolescents with autism and other pervasive developmental disorders (PDDs) yielded much less favorable results. Fluoxetine dosing was initiated at 25 mg/kg every other day and increased by 25 mg/day as tolerated. Only 1 of the 18 fluoxetine-treated subjects exhibited a favorable response, while 14 exhibited adverse effects, raising the possibility that both the efficacy and the tolerability of fluvoxamine may be lower in younger patients than in adults (McDougle et al., 2000).

A double-blind crossover trial of low-dose fluoxetine vs. placebo in 45 children and adolescents with ASD yielded more encouraging results. Fluoxetine treatment was initiated with a relatively low dose of 2.5 mg/day, which was then titrated over 4 weeks based on therapeutic response and side effects up to a maximum dose of 0.8 mg/kg, with treatment maintained for an additional 4 weeks. This was followed by a 4-week washout period and an 8-week double-blind crossover trial. Thirty-four subjects completed both phases of the trial. Significant improvement was found in repetitive behaviors as scored on the Children's Y-BOCS. There was no significant increase in adverse events as compared to placebo (Hollander et al., 2005).

A recent large, multi-center trial examined 149 children and adolescents with ASD who were randomized to receive either the SSRI citalopram or placebo for 12 weeks (King et al., 2009). Participants had a CGI illness severity rating of at least moderate and a score of at least moderate on the Children's Y-BOCS. Dosing was initiated at 2.5 mg/day and gradually escalated to a maximum of 20 mg/day as tolerated. Although approximately a third of the participants in the citalopram group were classified as responders based on ratings of "much improved" or "very much improved" on the CGI scale, this did not differ significantly from the percentage of subjects "responding" to placebo therapy. In addition to the lack of significant overall improvement on the CGI scale, there was no significant benefit over placebo with respect to compulsive and repetitive behaviors as measured by the CY-BOCS or the Repetitive Behavior Scale-Revised. The high level of apparent response to placebo therapy in this study is not unusual in ASD clinical trials

(Sandler, 2005) and illustrates once again the necessity of placebo-controlled study designs for evaluating ASD pharmacotherapies. Notably, the results from this study are at variance with previous open-label studies that reported benefits for citalopram in ASD (reviewed by Posey et al., 2006). In addition, adverse events were significantly more frequent overall with citalopram than with placebo, with significant increases in energy level, impulsiveness, impaired attention and concentration, hyperactivity, stereotypy, and insomnia.

Although favorable reports for other SSRIs have been reported in open-label studies (reviewed by Posey et al., 2006), these are of doubtful value for the reasons discussed above. The fact that the largest, most comprehensive study to date failed to find a benefit of a SSRI for the specific indication for which these agents are most commonly prescribed in ASD casts doubt on the value of this class of medications in ASD. Indeed, aside from one study of fluvoxamine in adults (McDougle et al., 1996) and one of fluoxetine in children (Hollander et al., 2005) (discussed above), there is little to support the use of SSRIs in ASD. Moreover, even in the studies that have reported favorable results, improvement in core symptoms has been found to be variable and generally rather modest, providing little support for the hypothesis that abnormalities in serotonin systems play a central role in autism.

In contrast to SSRIs, the atypical antidepressant tianeptine has been reported to enhance uptake of serotonin and has been proposed to act by restoring neuroplasticity (Costa e Silva, 2004). A double-blind crossover trial of tianeptine vs. placebo in 12 autistic children reported modest but statistically significant improvements after 6 weeks of treatment with respect to ABC measures of irritability, hyperactivity, inadequate eye contact, and inappropriate speech. The major side effects noted were drowsiness and decreased activity (Niederhofer et al., 2003). Larger studies of this agent in ASD populations would be useful.

Stimulants

After antidepressants, stimulants constitute the second most widely prescribed class of therapeutic agents for children with ASD (Oswald and Sonenklar, 2007). The primary rationale for the use of stimulants in ASD is to reduce symptoms of hyperactivity and irritability, based on their effectiveness in treating behavioral symptoms of attention-deficit hyperactivity disorder (ADHD) (Findling, 2008).

Quintana et al. reported a double-blind, placebo-controlled study of 10 autistic children. The study employed a randomized crossover design in which subjects received placebo (2 weeks) or methylphenidate (10 mg twice daily for the first week, 20 mg twice daily for the second week) in random order. A modest but statistically significant improvement over placebo was noted in ABC measures of hyperactivity, irritability, and stereotypic behavior, with no significant increase in adverse effects (Quintana et al., 1995).

Handen et al. (2000) reported a double-blind, placebo-controlled study of methylphenidate in 13 children with ASD, aged 5.6–11.2 years. The study employed

a crossover design in which subjects were treated for 7 days each in random order with placebo and two doses (0.3 or 0.6 mg/kg, two to three times per day) of methylphenidate. Eight (63%) of the subjects exhibited a favorable response to methylphenidate, as evaluated by the Hyperactivity Index of the Conners Teacher Rating Scale (Goyette et al., 1978). Significant improvement was noted in ABC measures of hyperactivity and inappropriate speech, but there was little indication of improvement in global symptoms of autism. Five (38%) of the children exhibited significant increases in side effects, which included social withdrawal, sadness, and irritability and which in three cases were severe enough that treatment had to be prematurely terminated. A caveat regarding this study is the short duration of each treatment phase and a relatively high frequency of adverse effects reported during the placebo phase of treatment, which could have impaired the ability to detect genuine adverse effects of methylphenidate.

A double-blind, placebo-controlled crossover trial of methylphenidate in 66 children with ASD who exhibited interfering symptoms of hyperactivity/impulsiveness found significant improvement on the ABC hyperactivity subscale, but little evidence of improvement in other ASD symptoms (irritability, lethargy/social withdrawal, stereotypy, and inappropriate speech). Subjects received three doses of methylphenidate (0.125–5 mg/kg, three times daily) and placebo for 1 week each in random order, and responders underwent an additional 8-week open-label continuation. Approximately half of the children were rated as responders, which is lower than typically reported for children with a primary diagnosis of ADHD. Moreover, despite conservative dosing, 18% of the subjects exited the study due to adverse effects, primarily increased irritability. Other adverse effects included difficulty in falling asleep, decreased appetite, and emotional outbursts. In addition, there was significant worsening on the lethargy/social withdrawal measure at the highest dose (Research Units on Pediatric Psychopharmacology Autism Network, 2005a).

In contrast, a large, retrospective chart review of 194 children and adolescents with PDD (Stigler et al., 2004) concluded that psychostimulants (principally methylphenidate) were poorly tolerated, with a high incidence (57%) of adverse effects, primarily agitation. Patients with Asperger syndrome were noted as significantly less likely to exhibit adverse effects. The origin of the apparent discrepancy with smaller controlled trials is not clear, but it is notable that whereas the controlled studies involved short-term psychostimulant treatment, the mean duration of treatment with methylphenidate in this retrospective study was 545 days, so it is possible that the incidence of adverse effects may rise with long-term treatment. On the other hand, given the lack of placebo controls in this study, it is possible that psychostimulants may have received the blame for symptoms that would have arisen even in the absence of pharmacotherapy. Another difference is that whereas the subjects in the controlled studies were not receiving other medications, 18% of the cases reviewed by Stigler et al. involved the concomitant use of other psychoactive medications. While such use was positively correlated with a favorable response to methylphenidate, it might also have contributed to the high incidence of adverse effects.

Taken together, these studies argue that methylphenidate may be of some value in treating hyperactivity in a subgroup of children with ASD, but that adverse reactions may limit its usefulness. In general, as with other pharmacological therapeutic strategies, evidence of safety and benefits of long-term treatment with methylphenidate is lacking.

Other stimulants used in treatment of autism include dextroamphetamine, dexmethylphenidate, modafinil, and pemoline (Oswald and Sonenklar, 2007). Aside from a small, early trial of dextroamphetamine that reported poor results (Campbell, 1975), these agents have not been evaluated in controlled trials with ASD subjects.

Atomoxetine is an ADHD medication that is considered to be nonstimulant. Its primary mode of action is thought to be inhibition of the presynaptic norepinephrine transporter (Findling, 2008). A double-blind crossover trial of atomoxetine vs. placebo was conducted with 15 children and adolescents who exhibited ASD with prominent ADHD symptoms. Participants received placebo and atomoxetine in random order, with the two 6-week treatment phases (3 weeks of titration up to a maximum daily dose of 1.4 mg/kg/day, 3 weeks continuation) separated by a 1-week unblinded washout period. Atomoxetine produced significant improvement over placebo on the hyperactivity and lethargy/social withdrawal ABC subscales. The primary significant adverse events noted were transient mild stomach upset or nausea/vomiting (which occurred in all subjects) and fatigue. Both are typical side effects commonly observed with treatment of ADHD with atomoxetine (Gibson et al., 2006), and only one patient had to terminate the study early due to adverse events. There was also a nonsignificant worsening trend in measures of stereotypy and compulsive behavior. The incidence of adverse effects compares favorably to that observed with methylphenidate, and while these results need to be confirmed in larger studies, they suggest that atomoxetine may offer a viable alternative for treatment of hyperactivity symptoms associated with ASD. Two large placebo-controlled studies of atomoxetine for treatment of hyperactivity associated with ASD are underway (clinicaltrials.gov).

Antipsychotics

Antipsychotics constitute the third most widely prescribed class of medications for treatment of ASD, with 23% of ASD patients receiving an antipsychotic in 2002 (Oswald and Sonenklar, 2007). A double-blind, placebo-controlled crossover study of 32 children with autism and mental retardation found that haloperidol at a mean dose of 1.12 mg/day produced significant improvements on the CGI scale and significantly reduced CPRS measures of withdrawal, stereotypies, hyperactivity, abnormal object relationships, fidgetiness, negativism, angry affect, and negativity of affect. In this study, the dosage was individually titrated until positive effects or untoward effects were seen, with a mean optimum haloperidol dose of 0.05 mg/kg/day. Although adverse effects at optimal dose were reported not

to differ significantly from placebo at optimal doses, acute dystonic reactions or excessive sedation was commonly observed with haloperidol during dosage regulation (Campbell et al., 1982; Anderson et al., 1984). A subsequent study of 60 children treated for 6 months with haloperidol followed by a 4-month placebo/drug withdrawal phase reported a high frequency of reversible dyskinesias associated with either administration or withdrawal of the drug (Perry et al., 1989). Moreover, a long-term longitudinal study of 118 autistic children treated with haloperidol found that a third of the subjects developed dyskinesias, suggesting that the risk of extrapyramidal side effects rises with prolonged haloperidol treatment (Campbell et al., 1997).

Combination therapy with haloperidol and cyproheptadine, an antagonist of H1 histamine receptors and 5-HT_2 serotonin receptors, was compared to haloperidol plus placebo in a double-blind, parallel-group trial in 40 autistic children and adolescents. Participants were randomly selected to receive haloperidol (titrated up to 0.05 mg/kg/day) plus either cyproheptadine (titrated up to 0.2 mg/kg/day) or placebo. Both groups received the anticholinergic drug biperiden (0.04 mg/kg/day) as prophylaxis against extrapyramidal symptoms. The cyproheptadine-treated group exhibited significantly greater improvement over the 8-week course of the trial on both the ABC-Community and the CARS (relating to people and verbal communications) scales. The incidence of adverse effects with cyproheptadine was lower by a statistically insignificant margin as compared to haloperidol alone (Akhondzadeh et al., 2004). This result suggests that cyproheptadine may be a promising agent for treatment of autism and that additional clinical trials are indicated to confirm this result and to assess its effectiveness in other therapeutic regimens.

Based on the expectation of lower risk of extrapyramidal side effects, current usage favors atypical antipsychotics, which combine 5-HT_2 receptor and dopamine D_2 receptor antagonism, over first-generation antipsychotics such as haloperidol, which are principally dopamine receptor antagonists. Atypical antipsychotics, primarily risperidone and olanzapine, are among the best established pharmacotherapies for autism (Stachnik and Nunn-Thompson, 2007; Chavez et al., 2006), and risperidone is the only drug to receive FDA approval for treatment of behavioral symptoms associated with autism in children. Risperidone exhibits high affinity as an antagonist of 5-HT_{2A} serotonin receptors, with somewhat lower affinity for dopamine D_2 receptors.

In a head-to-head double-blind comparison of risperidone to haloperidol, 30 children and adolescents were randomly assigned to receive 10 weeks of treatment with haloperidol or risperidone. Dosing was initiated at 0.01 mg/kg/day for both agents and individually titrated up to a maximum of 0.08 mg/kg/day. The risperidone and haloperidol treatment groups exhibited comparable improvement in autistic symptoms on the CGI scale and RF-RLRS sensory, social and affect subscales, and risperidone was significantly superior to haloperidol on the RF-RLRS sensory motor and language subscales, ABC scale, and the Turgay DSM-IV scale. There were no serious adverse effects reported in this study, but a significant increase in the Chouinard Extrapyramidal Symptoms Rating Scale was observed with haloperidol, but not with risperidone (Miral et al., 2008).

The effectiveness of risperidone in autistic adults was examined in a 12-week randomized, parallel-group, double-blind trial in 31 adults with autism or pervasive developmental disorder not otherwise specified (PDD-NOS). Risperidone was started at 1 mg/day and titrated individually up to a maximum of 10 mg/day. This study found significant improvement over placebo with respect to overall autistic symptoms on the CGI and RF-RLRS, with reductions in repetitive behavior as rated on the Y-BOCS and in self-injurious and aggressive behavior as rated on the Self-Injurious Behavior Questionnaire. Significant improvement was also reported on the RF-RLRS sensory motor behaviors subscale, but there was no significant improvement in measures of social relationships, affectual relationships, sensory responses, or language. Eight of 14 risperidone subjects were categorized as responders, compared to none in the placebo group. Side effects included sedation and weight gain (McDougle et al., 1998).

The most comprehensive evaluation of risperidone in autism to date has been an 8-week randomized, double-blind, parallel-group study of 101 autistic children and adolescents conducted by the Research Units on Pediatric Psychopharmacology (RUPP) Autism Network (Arnold et al., 2000). This study reported significant improvement over placebo on the ABC irritability subscale, with 69% of the subjects in the risperidone group (compared to 12% in the placebo group) exhibiting a favorable response at a mean dose of 1.8 mg/day (range = 0.5–3.5 mg). Significant improvement was also observed on the stereotypy and hyperactivity subscales, but scores on the social withdrawal and inappropriate speech subscales were not significantly improved (McCracken et al., 2002). Risperidone was also found to be superior to placebo on multiple subscales of a modified, parent-rated version of the RF-RLRS, with significant improvement on subscales for sensory motor behaviors, affectual reactions, and sensory reactions. No significant improvement was detected with respect to impaired social relatedness and language (McDougle et al., 1998). Significant improvement relative to placebo was also found on the compulsion subscale of the Children's Y-BOCS and in the maladaptive behavior domain of the Vineland Adaptive Behavior Scales (McDougle et al., 2005). Benefits were sustained in a 16-week open-label continuation (Aman et al., 2005), and a significant worsening of symptoms was detected when risperidone responders were switched to placebo in a subsequent double-blind discontinuation trial (RUPP Autism Network, 2005b). Although adverse effects were common, they were mostly mild. No cases of dyskinesia were observed over the entire 6-month duration of the study either in risperidone-treated subjects or in subjects in whom risperidone was discontinued (RUPP Autism Network, 2005b). The most prominent adverse effect was excessive appetite, which was rated moderate or severe in 33% of subjects, compared to 9.6% of the placebo group, as well as significant weight gain (Aman et al., 2005; Martin et al., 2004). Sedation, manifested as difficulty in waking and tiredness during the day, was also reported, although this appeared to decline with sustained treatment. Other significantly increased adverse effects included excessive salivation and dizziness (Aman et al., 2005).

An 8-week randomized, double-blind trial of risperidone at a mean dose of 1.2 mg/day in 79 children with PDD (Shea et al., 2004) reported significant

improvement on multiple scales of the ABC, including irritability, hyperactivity/noncompliance, inappropriate speech, lethargy/social withdrawal, and stereotypic behavior, as well as improvement in Conduct Behavior Rating Form measures, including the conduct problem, hyperactive, insecure/anxious, and overly sensitive scales. Overall, 54% of the risperidone group was rated as much or very much improved vs. 18% of the placebo group. Risperidone was found to be well tolerated; the most common adverse effect was somnolence (72.5% vs. 7.7% with placebo), but this was reported to be largely manageable by adjustments in dosing schedule or dose. Weight gain and increased appetite were also observed, but were not particularly prominent in this study. A subgroup analysis indicated that risperidone was similarly effective in the subgroup of study participants diagnosed with autistic disorder, with significant improvements in measures of irritability and hyperactivity/noncompliance (Pandina et al., 2007).

To determine whether earlier pharmacological intervention with risperidone would result in more substantial improvement in core symptoms of autism, a 6-month trial of low-dose (0.5–1.5 mg/day) risperidone was carried out in preschool children diagnosed with autism or PDD-NOS who were also receiving behavioral therapy (Applied Behavior Analysis) (Luby et al., 2006). Risperidone was reported to be well tolerated in this age group, with the most common averse events being transient sedation, increased appetite, and hypersalivation. This study failed to detect significant improvement over placebo with respect to core social and behavioral features as measured by the CARS, although a post hoc reanalysis of the data suggested that there might have been some improvement in the group that received risperidone. Nevertheless, the study did not support the expectation that earlier intervention with risperidone would yield dramatic improvements in outcome.

Based on these studies, risperidone appears to be effective in improving some autism-associated behaviors in some patients, but with little evidence of improvement in core features of impaired social relatedness and language. Risperidone is reasonably well tolerated in the short term, with increased appetite and weight gain being the side effects most likely to limit its use. Given the absence of evidence for substantial improvement in core symptoms of autism, a major rationale for the use of risperidone is to reduce disruptive behaviors in order to facilitate learning and behavior therapy. This raises concerns as to the impact of possible side effects of risperidone, and sedation in particular, on cognition. This concern has been addressed directly by Aman et al. (2008), who performed cognitive assessments on a subset ($n = 38$) of participants of the RUPP study (McDougle et al., 1998) who had scorable measures at baseline and at least one subsequent visit. No decrement in cognitive performance was found for the risperidone group, and in fact there was a trend toward improvement, although the improvement was not statistically significant after adjustment for multiple comparisons.

Other atypical antipsychotics commonly prescribed for treatment of autism include olanzapine, quetiapine, ziprasidone, and clozapine (Oswald and Sonenklar, 2007). Placebo-controlled trials of these agents in ASD populations have not been reported, with the exception of a small pilot study of olanzapine in which three of six children treated with olanzepine were rated as responders, compared to one of five in the placebo group (Hollander et al., 2006b). Open-label studies (reviewed by

Posey et al., 2008) with these agents have been generally consistent with results for risperidone and suggest that the therapeutic benefits of risperidone are likely shared by other drugs in this class. Placebo-controlled trials in ASD patient populations are in progress for olanzapine, sertraline, and aripiprazole (clinicaltrials.gov).

It is generally presumed that the risk of extrapyramidal side effects is less with atypical antipsychotics such as risperidone than with first-generation antipsychotics. Evidence to support this is somewhat scanty, and there is some indication that atypical antipsychotics may carry an elevated risk of extrapyramidal side effects in pediatric populations (Sikich et al., 2004). As noted above, a head-to-head comparison of haloperidol to risperidone found a lower incidence of dyskinesias with risperidone (Miral et al., 2008); however, the difference was not statistically significant. Although most studies have reported minimal problems at doses of risperidone used to treat maladaptive behaviors in PDDs, these have been relatively short-term studies of under a year in duration, and evidence suggests that as with haloperidol (Campbell et al., 1997), the risk of extrapyramidal side effects from atypical antipsychotics increases with duration of treatment (Laita et al., 2007). Other safety concerns relate to the tendency of this class of agents to produce weight gain in some patients, raising concerns that long-term treatment could increase the risk of metabolic and cardiovascular disorders.

Anticonvulsants

In 2002, approximately 15% of patients with an ASD diagnosis were treated with an anticonvulsant medication. In part, this reflects the fact that epilepsy is a frequent comorbidity in autism (Canitano, 2007), but 74% of the patients who received anticonvulsant treatment did not have a diagnosis of epilepsy (Oswald and Sonenklar, 2007).

Although originally developed as antiseizure medications, anticonvulsants have come into expanded usage as mood stabilizers. Clinical trials support the efficacy of a variety of anticonvulsants in regulating mood in adults with bipolar disorder (Soares-Weiser et al., 2007), and in 1995, valproate became the first anticonvulsant to be FDA approved for the treatment of bipolar disorder. Evidence supporting the safety and efficacy of these agents in treating psychiatric disorders in children is more limited, but they are widely used (Lopez-Larson and Frazier, 2006). Evidence for the effectiveness of anticonvulsants in treating symptoms of autism other than epilepsy is limited.

An 8-week placebo-controlled study in 20 subjects with PDD and symptoms of aggression toward self, others, or property found that 12 of 16 subjects treated with valproate exhibited a clinical response. Nevertheless, the effect of valproate was not statistically significant on any of the response measures (ABC-Community Scale; Overt Aggression Scale; CGI-Improvement subscale), because, as is often the case with clinical trials involving PDD, the placebo group also showed substantial improvement, with 8 of 14 subjects "responding" to placebo, even though the study design included a 1-week placebo run-in intended to eliminate placebo responders (Hellings et al., 2005). In contrast, another small 8-week trial in 13 subjects with

ASD found that valproate (divalproex) was significantly better than placebo in treatment of repetitive behaviors as measured on the Children's Y-BOCS (Hollander et al., 2006a).

A double-blind study of the anticonvulsant levetiracetam in 20 autistic children and adolescents failed to detect a significant difference between levetiracetam and placebo with respect to global improvement (CGI) or measures of repetitive behaviors (Children's Y-BOC) and/or hyperactivity (Conners Rating Scales-Revised) (Wasserman et al., 2006).

Based on an open-label study that reported that autistic children with intractable epilepsy who were treated with the phenyltriazine antiepileptic drug lamotrigine exhibited improvements in autistic symptoms unrelated to seizure (Uvebrant and Bauzienè, 1994), an 18-week double-blind, parallel-group study was conducted with 28 autistic children. Dosing was initiated at 0.5 mg/kg/day and gradually increased over the course of 4 weeks to 5.0 mg/kg/day, which was maintained by 4 weeks, followed by a 2-week tapering off period. Although the lamotrigine-treated subjects exhibited improvements in autistic symptoms as measured by the Autism Behavior Checklist and the ABC, they did not differ significantly from the placebo group, which exhibited a similar degree of improvement. Ratings on the Vineland, CARS, and Pre-Linguistic Autism Diagnostic Observation Schedule also failed to yield evidence of a clinical benefit (Belsito et al., 2001). The results thus do not support the hypothesis that lamotrigine is useful for treating autistic symptoms other than seizures.

Other anticonvulsants frequently prescribed to autistic patients include topiramate, gabapentin, and carbamazepine (Oswald and Sonenklar, 2007). Evidence from controlled studies for the use of these agents for treatment of autistic symptoms other than seizures is not available, although results from open studies with topiramate (Canitano, 2005; Hardan et al., 2004) suggest that this drug is worthy of further investigation. A placebo-controlled trial of oxcarbazepine in childhood autism is underway (clinicaltrials.gov).

Alpha$_2$ Adrenergic Antagonists

Alpha$_2$ adrenergic agonists such as clonidine and guanfacine, which decrease sympathetic tone via central effects and feedback effects on norepinephrine release and which tend to produce an overall sedating effect, are used primarily to treat hyperaroused behavior, sleep disturbances, and tic-like behavior in autistic patients (Oswald and Sonenklar, 2007). Evidence for the efficacy of this class of drugs in autistic patients is limited.

A small double-blind, placebo-controlled crossover study in seven children and two adults with autistic disorder and hyperarousal behavior found significant improvement relative to placebo with 0.5 mg/kg/day transdermal clonidine on the CGI scale. The primary adverse effects were fatigue and sedation (although it was stated that these declined over the course of the 4-week treatment period) and skin irritation from the transdermal patch. Blood pressure was significantly reduced

during the clonidine treatment phase, but clinical signs of hypotension were not observed (Fankhauser et al., 1992).

A recent open-label retrospective study of clonidine in 19 children with ASD reported that clonidine reduced sleep latency and night awakenings and that clonidine (0.05 mg/day, gradually increased to 0.1 mg/day) was generally well tolerated by most patients over the extended duration (from 6 to over 24 months) of the study, although a high incidence of problems relating to the transdermal patches resulted in most of the patients being switched to an oral preparation (Ming et al., 2008).

A small randomized crossover study examined 11 children with autistic disorder or PDD-NOS and with ADHD symptoms who were treated for 6 weeks each with placebo and guanfacine in random order. Treatment was initiated at 0.5 mg/day and gradually increased up to 3 mg/day or the maximum tolerated dose. Guanfacine was superior to placebo with respect to the CGI Global Improvement score and on the parent- and teacher-rated ABC hyperactivity subscales, with five of the subjects considered to be responders. No significant difference between guanfacine and placebo was detected on other ABC scales of autistic behavior. Side effects were not significantly different than placebo, but three subjects were unable to tolerate the maximum dose, and five of the subjects experienced drowsiness or lethargy (Handen et al., 2008).

Melatonin

Abnormal sleep patterns are frequent in autism and constitute a major source of stress for caregivers (Wiggs and Stores, 2004). Melatonin is a pineal hormone that regulates sleep. A double-blind crossover study (10 days in each phase) of sustained-release melatonin (5–15 mg) vs. placebo in 51 children with a variety of neurodevelopmental disabilities found significant improvement in sleep latency and total nighttime sleep, increasing average total nighttime sleep and reducing sleep latency by about half an hour (Wasdell et al., 2008). Benefits were also significant in the subgroup of 16 patients with ASD. Benefits appeared to be sustained in a 3-month open-label extension. Melatonin was well tolerated, without serious adverse events attributable to the drug. A smaller double-blind crossover study of 11 children with ASD yielded similar results, with significant improvements in sleep latency, night awakenings, and total sleep time (Garstang and Wallis, 2006) The results indicate that melatonin is likely to be beneficial in the treatment of sleep disturbances in ASD patients.

Naltrexone

Interest in the opiate antagonist naltrexone as a potential treatment for autism is long-standing and derives largely from an analogy between the symptoms of autism and the effects of opiate administration, leading to the hypothesis that autism might be a consequence of hypersecretion of endogenous opiates (Deutsch, 1986).

In a double-blind trial, 18 children with autistic disorder and mental retardation were randomized to receive either placebo or 0.5–1.0 mg/kg/day naltrexone for 3 weeks following a 2-week placebo run-in period. This study found a significant therapeutic effect of naltrexone for a CPRS measure of hyperactivity, but failed to demonstrate significant improvement in language or in performance in a test of discriminative learning (Campbell et al., 1990). A similar study in a larger group of 41 autistic children yielded comparable results with a significant reduction in hyperactivity, but no improvement in the core symptoms of autism or in discrimination learning. Naltrexone was generally well tolerated, with a small number of children in the naltrexone group exhibiting sedation or nausea (Campbell et al., 1993).

A double-blind crossover study of 10 autistic children found no significant difference relative to placebo in improvement of autistic symptoms as measured by the CPRS after a 30-day treatment with low-dose (0.5 mg/kg/day) naltrexone, although it is possible that sensitivity of this study may have been reduced by the lack of a washout period between the two phases of this crossover study, potentially allowing carryover of a naltrexone effect (Bouvard et al., 1995).

To test the hypothesis that elevated endogenous opiate levels contribute to autism and/or to self-injurious behavior, 33 adult subjects with autism and/or self-injurious behavior were treated for 4 weeks with 50 or 150 mg/day naltrexone in a double-blind, placebo-controlled crossover trial. Naltrexone was not superior to placebo with respect to either frequency of self-injurious behavior or CGI and ABC measures of autistic symptoms (Zingarelli et al., 1992).

A double-blind crossover study of 20 autistic children treated with a single, relatively high dose of naltrexone (40 mg/day, corresponding to 1.48–2.35 mg/kg) found a modest but statistically significant improvement in hyperactivity and temper tantrums, but failed to detect a significant improvement in social behavior (Willemsen-Swinkels et al., 1995). In what appears to have been the same group of autistic children, the same investigators evaluated the response to a lower dose of naltrexone (20 mg/day) using a crossover study design with two 4-week naltrexone/placebo treatment phases separated by a 4-week washout period (Willemsen-Swinkels et al., 1996). In this study, a significant improvement was noted by teachers, but not parents, on the ABC hyperactivity and irritability subscales, but there was no significant improvement in social interactions. Seven of the subjects were identified as individual responders, and six of these were followed in a subsequent 6-month open-label extension of naltrexone therapy. No indication of improvement in core symptoms of social interaction, communication, or stereotypic behavior was observed, and parents did not request to continue naltrexone treatment (Willemsen-Swinkels et al., 1999).

A double-blind crossover study of naltrexone (1 mg/kg/day, 2 weeks) in 13 children with autistic disorder found significant improvement over placebo with respect to both parent and teacher ratings on the CGI scale, the impulsivity–hyperactivity factor of the Connor Rating Scale, and restlessness on the Naltrexone Side Effect Rating Scale. Overall, 8 of the 13 subjects demonstrated modest improvement during the naltrexone treatment phase. No significant adverse effects of naltrexone were observed, although the bitter taste of naltrexone was a problem for some children.

The use of an initial 2-week placebo run-in/baseline phase in this study may have helped to minimize confounding placebo effects (Kolmen et al., 1995). A follow-up study by the same research group extended this study by adding an additional 11 subjects. While improvement with naltrexone was not statistically significant in these 11 subjects considered alone, with only 3 subjects showing a favorable response, the results from the pooled group of 24 children continued to show a highly significant therapeutic benefit. The follow-up study also examined measures of learning, but found no significant improvement over placebo (Kolmen et al., 1997). Similarly, there was no significant effect of naltrexone on communication skills (Feldman et al., 1999).

Overall, the results indicate that naltrexone has little if any therapeutic efficacy with respect to learning or core symptoms of autism and argue against the hypothesis that overproduction of endogenous opioids plays a major role in autism. Nevertheless, the results indicate that naltrexone is generally well tolerated and may offer modest benefits primarily for treatment of hyperactivity associated with autistic disorder.

Acetylcholinesterase Inhibitors

Acetylcholinesterase inhibitors have been shown to provide modest improvement in dementia associated with Alzheimer disease (Birks, 2006). Results from case reports and a single placebo-controlled study (reviewed by Yoo et al., 2007) suggest that the second-generation cholinesterase inhibitor donepezil may be useful in the treatment of autism, with some evidence of improvement in language. An open-label trial of galantamine in 13 autistic children reports improvement in measures of irritability, hyperactivity, and social withdrawal (Nicolson et al., 2006), and a case report of 3 adult autistic patients claims improvements in verbal fluency (Hertzman, 2003). A brief account of a placebo-controlled crossover study of 20 autistic children reported galantamine to be superior to placebo in improving ABC measures of hyperactivity, inadequate eye contact, and inappropriate speech (Niederhofer et al., 2002). Similarly, an open-label trial in 32 autistic patients reported improvement in overall autistic behavior and expressive speech (Chez et al., 2004). Although the quality of the evidence is not high, the suggestion of improvements in communication is interesting, as this is an area where currently used medications show no clear efficacy. Placebo-controlled trials of donepezil and galantamine are underway (clinicaltrials.gov).

NMDA Receptor Antagonists

Amantadine has its primary medical use as an antiviral drug in the treatment of influenza by virtue of its ability to block a viral ion channel that is required for uncoating of the viral particle. In addition, it possesses weak channel blocking activity at NMDA receptors. A double-blind trial of amantadine (2.5 mg/kg for 1 week,

then 5 mg/kg for 3 weeks, somewhat lower than the dosing used for influenza) vs. placebo in 39 autistic subjects aged 5–19 years found no significant overall benefit relative to placebo with respect to parental ratings on the ABC-Community version, although significant improvement was observed in investigator ratings on the hyperactivity and inappropriate speech subscales, suggesting that there may have been a small benefit. Amantadine was well tolerated, with the most common adverse effect being insomnia (King et al., 2001).

The weak NMDA receptor uncompetitive antagonist memantine has been found to provide a modest benefit in the treatment of dementia associated with Alzheimer disease and is FDA approved for that use (McShane et al., 2008). Excitotoxicity resulting from NMDA receptor over-activation has been proposed as a mechanism of neuronal damage in Alzheimer disease as well as other neurodegenerative diseases and developmental disorders, and memantine might reasonably be expected to be neuroprotective, but this mechanism is unlikely to account for relatively rapid therapeutic effect of memantine. The mechanism whereby memantine improves cognition is not well understood, as NMDA receptor antagonists can also interfere with learning by blocking long-term potentiation (Parsons et al., 2007). Limited evidence suggests that memantine may provide some benefit in ASD (Zdanys and Tampi, 2008), but evidence from placebo-controlled trials is not available.

d-Cycloserine is a partial agonist at the glycine recognition site of the NMDA receptor. As activation of the glycine site is required for receptor activation, a partial agonist could in principle act as an NMDA receptor agonist or antagonist, depending on whether levels of endogenous glycine-site agonists (glycine and d-serine) are low or high; however, its clinical effects have generally been attributed to facilitation of NMDA receptor-mediated neurotransmission. Some clinical studies have reported d-cycloserine to be effective in improving negative symptoms of schizophrenia, but other studies have failed to reproduce this result, and a recent meta-analysis concluded that it is without benefit (Tuominen et al., 2006). Some studies have found d-cycloserine to be beneficial in treatment of OCD (Wilhelm et al., 2008; Kushner et al., 2007), but negative results have also been reported (Storch et al., 2007). A small single-blind pilot study reported significant improvement on the CGI severity rating and the ABC social withdrawal subscale in eight autistic patients after 6 weeks of treatment with d-cycloserine on an escalating dose protocol (0.7–2.8 mg/kg/day) (Posey et al., 2004). Although this study included a 2-week placebo run-in, it did not have a parallel placebo group control, and a clear trend toward improvement was evident during the placebo phase, so this result is suggestive at best. A larger, randomized, double-blind, placebo-controlled crossover trial, projected to include 80 children, is underway (clinicaltrials.gov).

Benzodiazepines

Individuals with ASD frequently exhibit elevated levels of anxiety (White et al., 2009; Gillott and Standen, 2007). Benzodiazepines are widely used for the treatment of anxiety, but there have been few clinical studies of the efficacy of this class

of agents in ASD populations. Oswald and Sonenklar report that in 2002, only 4.2% of ASD patients received at least one prescription for a benzodiazepine (Oswald and Sonenklar, 2007), suggesting that benzodiazepines are not regarded as particularly useful for treatment of anxiety in this patient population. Radioligand binding evidence suggests that the density of benzodiazepine-sensitive $GABA_A$ receptors in hippocampus and cerebellum is reduced in autism (Guptill et al., 2007; Blatt et al., 2001). One early case report describes paradoxical anxiogenic and aggressive responses to diazepam in seven autistic children (Marrosu et al., 1987); however, a more recent report describes the routine use of midazolam for preanesthetic medication of autistic children (van der Walt and Moran, 2001), indicating that benzodiazepines are effective for sedation in this group of patients.

Conclusion

Overall, the status of pharmacotherapy for autism remains disappointing. While a number of agents have been found to be somewhat useful in the treatment of ASD, evidence of efficacy with respect to core symptoms of impaired social interactions and communication is weak. The greatest success has been in treating problems of hyperactivity and irritability. It is notable that with the exception of the stimulants, most of the medications that have been found to be effective are to some extent sedative, raising the question of whether the therapeutic effects that have been observed are in fact specific to autism or simply a consequence of nonspecific sedation. The absence of clear effects on core symptoms means that pharmacology has thus far provided few clues as to the neurochemical basis of ASD.

Studies over the years of adult outcomes for children diagnosed with autism have been discouraging, with typically half or more being rated as having a “poor” or “very poor” outcome. A recent follow-up study of 48 individuals born during 1974–1984 and diagnosed as children with ASD suggests that there has been some progress, with none of the adult participants falling into the “very poor” category, but there clearly remains great room for improvement, as the majority of participants were reported to have difficulty managing day-to-day life. Notably, 39.5% of the participants were still taking prescription medication for behavior problems, the most common medication being risperidone (Eaves and Ho, 2008). This emphasizes the likelihood that medication will be lifelong for many patients with autistic disorder. Most clinical trials have been a few weeks in duration, and there remains uncertainty regarding the consequences of long-term therapy from childhood with psychoactive medications.

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Appendix: Lay Abstracts/Summary

Chapter 1: Autism Spectrum Disorders: Clinical and Medical Perspectives

Margaret L. Bauman

The autism spectrum disorders (ASD) are identified by behaviorally defined clinical features characterized by core symptoms of impaired social interaction, delayed and disordered communication/language, and isolated areas of interest. It is now recognized that individuals affected with ASD are heterogeneous in terms of the cause of their disorder and the degree to which each is affected functionally and neurobiologically. Since its initial description in 1943, a growing body of research has broadened our perspective of the disorder and has created an environment whereby early diagnosis and intervention has become the standard of care, with the realization that, in many cases, early identification and intensive therapies can lead to more positive developmental outcomes. With advancements in clinical care, has come the observation and appreciation of the fact that many ASD children, adolescents, and adults have medically relevant health-care issues that may go undetected due to atypical symptom presentation, the inability of the patient to describe or localize discomfort and often due to behavioral issues that can make physical examination challenging. However, many of these medical conditions are treatable, and if and when identified, may improve quality of life for the patient and his/her family and may broaden our understanding of the phenotypic expression of ASD, leading ultimately to a better defined genotypic sub-typing of individuals on the spectrum.

Chapter 2: The Male Prevalence in Autism Spectrum Disorders: Hypotheses on Its Neurobiological Basis

Flavio Keller and Liliana Ruta

The strong male prevalence of autism spectrum disorders represents a challenge to any neurobiological theory of autism. Current genetics does not offer clues to this

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phenomenon. The authors argue that understanding the mechanisms by which male and female sex hormones act during brain development to organize brain circuits that are involved in sexually dimorphic and/or lateralized cognitive, social, and emotional functioning will yield important clues to understand the etiology of autism. The internal environment of the fetus and the newborn baby is characterized by constantly changing sex hormone levels: although in the past decades important insights have been gained into the effect of hormonal exposure on the organization of brain circuits, there are still many contradictory findings that need to be reconciled. Some apparent difficulties are the differences in sex hormone metabolism between animal species, and the fact that androgen and estrogen pathways are metabolically linked together. It is possible that altered androgen and estrogen levels could both lead, via different pathways, to alterations of cognitive, linguistic, social, and emotional circuits. For example, testosterone has important effects on development of callosal connections, thus leading to differences in hemispheric specialization between males and females, while recent research suggests a strong link between estrogen pathways and pathways of the "social hormones," oxytocin and vasopressin.

In conclusion, we think that in the years to come the challenge will be to gain a deeper understanding of the complex relationships between androgens/estrogens and development/plasticity of specific neural circuits that appear to be critical in autism, in particular reciprocal connections between the cerebral cortex and structures such as the amygdala and the cerebellum.

Chapter 3: Neuroanatomical-Behavioral Correlates in Autism: A Working Hypothesis

Ricardo M. Vela

This chapter correlates emotions and attachment behavior in autism with the disturbed neuroanatomy found in the brains of these individuals. Neuropathological postmortem studies have consistently found abnormalities in the limbic system and the cerebellum of autistic individuals. Abnormal limbic structures include the amygdala, hippocampus, septal nucleus, and anterior cingulate cortex. In the cerebellum, abnormalities have been found in the cerebellar hemispheres and in the fastigial, emboliform, and globose nuclei.

Autistic individuals fail to activate the amygdala when required to interpret emotions in facial expressions. A normal amygdala is able to discern subtle social–emotional nuances and attach emotional significance to sensory input, something which appears to be deficient in persons with autism. Abnormal processing of fear by the amygdala during development may result in failure of autistic individuals to detect and avoid danger.

Normal septal nuclei, together with the anterior cingulate cortex, act to promote selective attachments to other humans. Autistic children show deficient attachment behavior and early failure of person-specific bonding. The anterior cingulate cortex is involved in processing and modulating the expression of emotional nuances,

which appears deficient in autistic individuals. It is capable of producing emotional sounds and the separation cry, in an attempt to seek comfort. The hippocampus, in concert with the medial hypothalamus and septal nuclei, prevents extremes in arousal and maintains quiet alertness. In addition, it has strong interactions with the amygdala in storing emotional reactions to events and recalling personal emotional memories, functions that appear to be impaired in autism.

Cerebellar abnormalities may result in disturbances in a neural network involved in the motivation and organization of emotion. Abnormalities in the cerebellar nuclei may play a role in the affective disturbance, abnormal language development, and inappropriate social and psychological behaviors in autism.

Development of limbic structures in normal infants, during the first year of life, follows a pattern that correlates with the development of attachment behavior. The amygdala becomes functional early in life, promoting indiscriminate attachments. The septal nucleus and anterior cingulate cortex functionally develop in the second half of the first year of life and correlate with specific attachments and fear of strangers. These developmental patterns appear disturbed in autistic infants.

The study of psychological-neuroanatomical correlates of autism can provide a new understanding of this perplexing disorder.

Chapter 4: The Morphology of Minicolumns

Alan Peters

The cerebral cortex is composed of minicolumns, which are vertically oriented arrays of neurons. The minicolumns can be demonstrated both physiologically and morphologically. Physiologically, the minicolumns become evident when a recording electrode is advanced through the cortex in a horizontal direction. It is then found that as the electrode is advanced there are shifts in the receptive field properties of neurons. These shifts occur every 50 microns or so, as the electrode moves from one minicolumn to the next one. The anatomical correlates of the minicolumns are neuronal units based on vertically organized groups of pyramidal neurons, whose apical dendrites cluster together as they ascend through the layers of the cortex. The clusters of apical dendrites define the central axes of the three-dimensional minicolumns, which are similar in size to the physiological ones. Recently, digitized images of vertically oriented Nissl stained sections have been used to determine if there are changes in organization of minicolumns in aging and in some behavioral disorders such as autism. In Nissl sections vertically oriented strings of neurons are seen to be separated by clear spaces and it is assumed that these spaces correspond to the locations of the clusters of apical dendrites seen in the three-dimensional reconstructions of minicolumns. By analyzing digitized images of the Nissl stained sections it has been found that there is some disorganization of the minicolumns as a consequence of aging, and that in Alzheimer's disease the minicolumns are almost completely disrupted. In autism the minicolumns are narrower than in control brains, suggesting that the minicolumns are more numerous than normal. Since

the minicolumns are generated as the pyramidal neurons migrate into the cortex along radial glia fibers, it is suggested that autistic changes in cerebral cortex must have their origins during development.

Chapter 5: The Developmental Neuropathology of Autism

Thomas L. Kemper

The cellular neuropathology of autism is well described and illustrated in several reports. This chapter focuses on the timing and mechanism of these pathological changes, an aspect of the pathology of the autistic brain not covered in detail in the prior reports. Particular attention is paid to the changes in the brain stem, the cerebellum, and the cerebral cortex. Although the molecular (genetic) mechanism of these diverse neuropathologies is unknown, it can be seen that there is broad diversity of these changes, with perturbations of several different developmental processes, rather than reliance on a single mechanism. The vast majority of these pathologies can be dated to the prenatal period. The final section explores the relationship of these prenatally derived pathologies, and perturbation of other postnatal developmental processes, to the well-documented abnormal postnatal brain growth. I conclude that, based on the available data, that none of these mechanisms seem likely to account for the abnormal postnatal brain growth.

Chapter 6: Understanding Alterations During Human Brain Development with Molecular Imaging: Role in Determining Serotonin and GABA Mechanisms in Autism

Diane C. Chugani

The purpose of this chapter is to present an approach to the understanding of chemical differences in the brains of children with autism and to use this information to design new treatments for autism. The approach is to utilize information about how the processes in the developing brain of an autistic child differs from those in typically developing children discovered through molecular imaging to design new pharmacological treatments to bring brain development in the autistic child back on course.

Chapter 7: Glutamic Acid Decarboxylase (GAD) as a Biomarker of GABAergic Activity in Autism: Impact on Cerebellar Circuitry and Function

Gene J. Blatt, Jean-Jacques Soghomonian, Jane Yip

Basic circuits in the brain involve excitatory and inhibitory mechanisms. Inhibition in the brain is primarily sustained by the inhibitory neurotransmitter,

gamma-amino-butyric acid (GABA). Neurons that use GABA as their neurotransmitter selectively express the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD). This review focuses on recent studies showing abnormalities in the expression of two GAD isoforms, GAD65 and GAD67, by cerebellar GABAergic neurons in postmortem brain tissue sections from individuals with autism. The region of the cerebellum involved in these studies is in the lateral hemisphere, a part of the neocerebellum, in the Crus II region. This region of interest was chosen because of its abundant inputs from the frontal lobe, a region that is important for motor and cognitive processing including high-order executive function and set-shifting behavior tasks. These studies suggest that inhibitory mechanisms may be deeply altered in autism. Specifically, Purkinje cells that are decreased in some autism brains have a large decrease in GAD67 mRNA levels; basket cells, a type of inhibitory interneuron in the molecular layer of the cerebellum contains increased GAD67 mRNA levels; dentate neurons, from the dentate nucleus, a type of deep cerebellar nuclei, contain decreased GAD65 mRNA levels, whereas a large inhibitory neuron type in the granular layer of the cerebellum, Golgi type II cells have normal GAD67 mRNA levels. This chapter describes possible alterations of the normal functioning of cerebellar circuitry which may impact motor and/or cognitive behaviors related to the autistic phenotype. The possibility that these changes could also be a causal role in the disease process is presented.

Chapter 8: Epigenetic Dysregulation of 15q11-13 $GABA_A$ Receptor Genes in Autism

Amber Hogart and Janine M. LaSalle

GABA is the major inhibitory neurotransmitter in the mammalian brain and defects in inhibition have been implicated in autism spectrum disorders. GABA inhibition is mediated through a variety of receptor subunit genes. Three $GABA_A$ receptor subunit genes, *GABRB3*, *GABRA5*, and *GABRG3,* are located on chromosome 15q11-13. In addition to $GABA_A$ receptor subunit genes, chromosome 15q11-13 contains genes that are expressed based on the parental origin of the chromosome, a process termed genomic imprinting. Due to imprinting, deletions of chromosome 15q11-13 result in two different disorders, Prader–Willi syndrome and Angleman syndrome, depending on the parental origin of the deletion. Additionally maternal duplications of this genomic region are observed in 1–3% of autism cases. Although each of these disorders is clinically distinct, there are similarities in phenotypes, such as seizures, that may be due to improper levels of $GABA_A$ receptor genes. Genetic studies of families with autism without a known genetic cause have found evidence that genetic markers in *GABRB3* are significantly associated with autism. Although genetic mutations have not been found in *GABRB3*, significantly reduced levels of GABRB3 protein were found in a majority of autism brain samples. Further studies have shown an abnormal expression pattern of only one parental copy of 15q11-13 $GABA_A$ receptor genes in a subset of autism samples. Since no evidence

was found for a direct genetic explanation for this abnormal gene expression, we hypothesize that epigenetic changes, heritable modifications to DNA, and the proteins that are involved in DNA packaging, may explain the expression abnormalities in autistic brain samples.

Chapter 9: Cholinergic Component of Autism Spectrum Disorder

Elizabeta B. Mukaetova-Ladinska, Jodie Westwood, Elaine K. Perry

Autism is a neurodevelopmental disorder characterized by impaired social skills, communication deficits, and repetitive behaviors. Alterations in a variety of transmitter signaling systems including serotonin, oxytocin, gamma-aminobutyric acid (GABA), glutamate, and acetylcholine have been reported in autistic individuals. These are potentially relevant to psychoneuropharmacological therapeutic strategies.

An interesting hypothesis regarding transmitter signaling in autism implicates an imbalance in the major executive excitatory and inhibitory impulses in the premature autistic brain, combined with defects in secondary neurotransmitter systems, to cause autistic traits. In this chapter we concentrate on the cholinergic changes investigated in autism based on brain bank tissue. There is significant reductions in muscarinic and nicotinic receptors in various neocortical areas, including frontal and parietal neocortex, thalamus, as well as cerebellum in autistic subjects. In contrast, the level of choline acetyltransferase is not altered, suggesting that the presynaptic cholinergic projections are spared. The cholinergic receptor changes could be caused by abnormal cortical neuronal morphology (including synaptic and dendritic abnormalities, as well as neuronal migrational arrest).

Although a wide range of drugs are used to ameliorate symptoms of autistic behavior, currently there is no effective treatment. We discuss novel treatments such as use of cholinesterase inhibitors and nicotinic receptor antagonists as intervention therapies for treatment of the cognitive and non-cognitive changes in the autistic spectrum disorders.

Chapter 10: Oxytocin and Autism

Peter Kirsch and Andreas Meyer-Lindenberg

Disturbed social interaction is a central feature of autism and is the source of many of the everyday impairments of patients, their relatives, and their caregivers. Therefore, it made and still makes sense to study the neurobiology of social function. In addition to helping us understand the disorder, this also holds promise for defining targets that could lead to new and more targeted treatments. An excellent

candidate for this approach is oxytocin (OT), a hormone that is central to social processes throughout the animal kingdom, especially for attachment to other so-called "prosocial behaviors." In this chapter, we first review the evidence for the neurobiological function of OT in humans, which is now coming together. Then, we review emerging evidence that OT and the neural and hormonal systems to which it is linked are abnormal in autism. Finally, we discuss attempts to use OT to treat autism.

Chapter 11: The Role of the Noradrenergic System in Autism Spectrum Disorders

David Q. Beversdorf

Drugs that decrease the activity of one of the chemical messengers in the brain, norepinephrine, are sometimes helpful for patients with autism-related conditions or autism spectrum disorders (ASD). Individuals with ASD have decreased flexibility in verbal problem solving. Activity of the norepinephrine system affects performance on such verbal problem-solving tasks in individuals without neurodevelopmental diagnoses in experiments using the norepinephrine beta-receptor blocker, propranolol. In individuals without neurodevelopmental diagnoses, response to propranolol depends on how hard the task is, and it is beneficial only for difficult problems. However, in other types of patients with altered norepinephrine or altered flexibility in thinking such as individuals under stress, patients with language loss due to stroke, and individuals in cocaine withdrawal, propranolol also benefits performance for simple problems. Due to decreased flexibility in verbal problem solving in ASD, preliminary evidence suggests that they also respond more favorably to propranolol for simple problems. Future research will be necessary to determine how this cognitive response relates to the clinical response to these agents to better determine if this is a potential mechanism for treatment in ASD.

Chapter 12: Oxidative Stress in Autism and Its Implications for Dopamine-Stimulated Phospholipid Methylation

Richard Deth, Christina Muratore, and Mostafa Waly

Neurons operate under unique redox conditions, increasing their vulnerability to oxidative stress, and recent studies provide evidence of oxidative stress and neuroinflammation in autism. Impaired methylation is a consequence of oxidative stress, mediated in major part by inhibition of the folate- and cobalamin-dependent enzyme methionine synthase. Since methionine synthase activity is essential for dopamine-stimulated phospholipid methylation, some symptoms of autism may reflect impairment of this process. For example, dopamine D4 receptor activation

plays an important role in gamma frequency synchronization of neural networks during attention, and autistic children display deficits in synchronization. This chapter reviews the metabolic events contributing to impaired methylation and examines the mechanisms by which they may contribute to neurodevelopmental disorders such as autism.

Chapter 13: Neuroligins and Neurexins: Synaptic Bridges Implicated in Autism

Craig M. Powell and Antony A. Boucard

Neuroligins and neurexins are molecules that bridge the gaps between one neuron and another at synapses in the brains of humans and other animals. Recent human clinical genetics studies have revealed mutations in the genes that code for neuroligins and neurexins in some patients with autism spectrum disorders. These mutations alter the function of these synaptic bridge molecules and thereby alter the function of synapses. This leads to abnormally altered communication between neurons in neuronal circuits of the brain. When these mutations in the human genes are reproduced in mice, the mice show abnormal social behaviors and other behaviors relevant to human autism. Using these genetic animal models, neuroscientists are now in a position to decipher the precise functional abnormalities in the brain that result from these autism-associated genetic mutations. Ongoing studies are using recordings from brain slices in these mouse models to understand the exact problems with brain function and to identify drugs or other treatments that can be used to normalize the function of brain synapses and circuits. Efforts to use this information to treat behavioral abnormalities in mouse models and ultimately in patients with autism are underway.

Chapter 14: The Neurophysical Chemistry of Autism: Postulates from Intelligence Modeling

Peter R. Bergethon

This chapter uses a unifying neuroscience theoretical paradigm called intelligence modeling (IM) and cognitive dynamics to connect the observable behaviors that both characterize and define the autism spectrum disorders (ASD) to the underlying anatomical and biochemical mechanisms from which those behaviors emerge. The underlying principles of intelligence modeling that are derived from systems, information, and cybernetic theories are described. This analysis is applied to the normal flow of information whose failure is hypothesized to give rise to the defining behaviors of autistic spectrum disorders. From the IM analysis the correlation between the energy needs required for computational solutions in the brain and the

resulting behaviors when the energy needs cannot be met is examined. The chapter concludes with a novel model of the underlying neural substrate for ASD and suggests neuroanatomical and physiochemical mechanisms that might be investigated to test this model.

Chapter 15: Pharmacological Treatment of Autism

Terrell T. Gibbs

Although there is little evidence that any medication reliably relieves the core symptoms of autism to a major degree, most autistic patients receive some form of pharmacotherapy, much of it off-label, with the primary therapeutic aim being to improve behaviors that interfere with behavioral and educational therapies. Because of the difficulties inherent in conducting randomized, placebo-controlled studies in pediatric populations, evidence of the benefits of this strategy is limited, and most such studies have been small and of short duration. Nevertheless, evidence from controlled studies indicates that the atypical antipsychotic drug risperidone is frequently effective for ameliorating symptoms of irritability, hyperactivity, social withdrawal, and stereotypic, repetitive behavior. There remains uncertainty as to the risk of serious adverse effects with long-term risperidone treatment. Weaker evidence supports the efficacy of antidepressant and stimulant medications, and evidence is ambiguous regarding the use of anticonvulsants as "mood stabilizers" in autistic patients. Promising results have been reported for a number of other medications, but additional controlled studies are needed to confirm the validity of these reports. There is little evidence that any currently employed pharmacotherapy appreciably improves impaired social interactions or communication in autistic individuals. There remains a great need for research into the long-term benefits and risks of pharmacotherapy in autism.

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