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REVIEW ARTICLE

The Neuropathology of Autism: A Review

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Abstract

Presented is a review of recent progress in the understanding of autism based on investigations of donated human brain tissue. Autism is a pervasive developmental disorder by the American Psychiatric Association's *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV) criteria, manifesting by age 3 and characterized by impairments in social interaction and communication, as well as restricted, repetitive, stereotyped patterns of behavior. Based on reported neuropathologic findings, these characteristic behaviors are clinical manifestations of both pre- and postnatal alterations. This review summarizes the current data obtained from postmortem brain studies in the areas of stereology, neurotransmitter systems/synaptic processes, molecular mechanisms, and neuroimmunology. In addition, we discuss current research strategies designed to facilitate translational research and maximize the yield of precious resources (e.g. the Autism Tissue Program), highlight barriers to research, and consider future trends.

Key Words: Asperger, Autism, Brain, Neurodevelopment, Neuropathology, Pervasive developmental disorder (PDD).

INTRODUCTION

Autism and Asperger disorder are 2 of 5 pervasive developmental disorders officially recognized by the American Psychiatric Association's *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV) criteria (1), often referred to collectively as autism spectrum disorders (ASDs). Children with symptoms who do not meet the specific DSM-IV criteria are diagnosed with pervasive developmental disorder, not otherwise specified (PDD-NOS). The remaining disorders in the group of ASDs are the rarer conditions of Rett syndrome and childhood disintegrative disorder, initially termed childhood schizophrenia.

These disorders are characterized by varying degrees of delay or impairment in 3 domains: communication skills, social interaction (e.g. poor eye contact), sensory hypo- and hyperreactivity, and a fourth area of rigid routines and repetitive/stereotyped behaviors. Various screening and diagnostic tools are used to identify autism; the assessment uniformly adopted as the research diagnostic standard by National Institutes of Health-funded autism centers is the Autism Diagnostic Interview-Revised (ADI-R) (2), with 153 coded responses in the protocol (42 of which are diagnostic algorithm items). Scores in the 3 domains and subset scores are used for psychometric analysis.

Prevalence studies in the United States and abroad estimate ASDs at 2 to 6 per 1,000 births, with over 1.5 million American children and adults affected (3). Based on high concordance rates in monozygotic twins and low concordance in dizygotic twins, autism is generally accepted as a complex genetic disorder with multiple loci contributing to the global phenotype. It is currently estimated that 15+ genes play a role in autism susceptibility. Furthermore, because maternal and not paternal duplications of chromosome 15q11-q13 have been linked to autism, epigenetic factors are also being explored (4, 5).

The etiology of autism is heterogeneous and may coexist with genetic disorders such as fragile X (6) and tuberous sclerosis (7) or with prenatal infections such as influenza (8) and cytomegalovirus (9). Epilepsy is a comorbid complication at rates of up to 33% (10), and there is a reported increase in mortality for those individuals with epilepsy (11). Autism, attention deficit hyperactivity disorder, and dyslexia share atypical cerebral asymmetry, an absence of the left hemisphere dominance for language (12).

In recent years, detailed studies of endophenotypes in the autism population have helped refine genetic analysis. Also called quantitative traits, endophenotypes are measurable psychometric characteristics. In autism, these traits include face recognition, processing emotional visual or auditory stimuli, visual tracking, eye gaze shifting, eye blink conditioning, age of first word or phrase, and "insistence on sameness." This last trait describes the prominence of some, but not all, autistic children to exhibit repetitive compulsions and have extreme difficulty with changes to their daily routine. This behavior was the focus of a large family genetic study that used a statistical method, called "ordered subset analysis," that sifts through complex genetic data and extracts genetic risk factors that affect only some of the total group. The researchers targeted families whose children scored high in the "insistence on sameness" category and discovered a strong link to the GABRB3 gene on chromosome 15q, where no such link had appeared before (13).

Stratification of patients with autism is also possible on the basis of dysmorphologies resulting from deviations in prenatal brain development. In a cohort of 260 individuals meeting the DSM-IV criteria for autism, a subset was defined

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by facial anomalies (41 of 260) and/or microcephaly (13 of 260). Combined into a group termed "complex" autism, this subset had a lower male to female ratio (3.2:1 vs 6.5:1), lower IQ, and generally poorer outcomes (14).

The persistent uncertainty and controversy about the potential causes of ASDs and the sporadic effectiveness of available therapeutic strategies drives the search for diagnostic biomarkers and more effective treatments. Postmortem research is a promising strategy to help define the pathogenesis of this disorder, and research in this area is expanding rapidly with technologic advances and greater numbers of clinically well-characterized donor brains. This review describes the current state of knowledge of autism neuropathology, available biologic resources for translational research, and future trends.

AUTISM BRAIN PATHOLOGY STUDIES

A neuropathologist performing a routine examination of a putative "autism" brain today generally relies on 2 landmark case studies totaling 15 brains (15, 16) and resource papers on brain weights (17, 18). A summary (19) of published case studies of approximately 40 brains from the first paper published in 1980 (20) through early 2004 describes an emerging pattern of increased cell packing in the limbic system, reduced numbers of Purkinje cells in the cerebellum, age-related changes in cerebellar nuclei and inferior olives, cortical dysgenesis, and increased brain size, especially in the *young* autistic child, as measured by head circumference, magnetic resonance image (MRI) brain volume, and postmortem brain weight.

These gross morphologic changes allude to complex neurodevelopmental mechanisms operating in autism. Further details are emerging from postmortem pilot explorations in 4 general categories: stereology, neurotransmitter systems/synaptic processes, molecular mechanisms, and neuroimmunology.

Stereology

Cerebral Cortical Measurements

The team of Margaret Bauman and Tom Kemper at Massachusetts General Hospital has made consistent and significant advances in defining features of young and adult autistic brains. They list as findings: 1) small cell size and increased packing density at all ages in the hippocampus, amygdala, and entorhinal cortex; 2) consistently reduced numbers of Purkinje cells primarily in the posteroinferior regions; and 3) age-related changes with reduced numbers of small, pale neurons in the vertical limb of the diagonal band of Broca, cerebellar nuclei, and inferior olive in adult brains compared with plentiful and abnormally enlarged cells in these areas in the pediatric donors with autism (21). Together, these observations are used in support of the idea of an ongoing pathologic process.

Another viewpoint argues that the study of older autistic brains reflects the outcome of early pathology, rather than an evolving process. A metaanalysis of MRI volume and postmortem fresh brain weight data of 55 postmortem brains (44 male) by age group (young child, age 2–5; older child, age 7–12; and adult age, 13–70 years) revealed a significant effect of age and both measurement types (volume and weight), with the youngest ages (2–5 years) showing the greatest deviation from normal (22).

Bailey et al reported cortical dysgenesis in 4 of 6 cases and observed increased neuronal density, neurons in the molecular layer, and abnormal laminar patterns (16). Two thirds of the cases had white matter abnormalities (ectopic grey matter in 3 cases and increased numbers of white matter neurons in one). A more recent comprehensive stereology on whole hemispheric sections of 6 pairs of age- and sex-matched autism and control brains investigating cortical neuron number and density found that, although there was no statistical group difference in mean *volumes* of either cortical gray matter, subcortical gray matter, white matter, or whole hemisphere, the autistic patients had significantly higher mean total neuron *numbers* in the cerebral cortex than the controls (19% increase, $p = 0.031$) with the strongest effect of increased numbers of neurons and density in the youngest (age 4) pair (23).

Cortical minicolumns have been measured in postmortem autism brains and compared with nonaffected controls. Minicolumns consist of approximately 80 to 100 neurons arranged radially like beads on a string and are believed to comprise the smallest level of functional organization in the cortex (24). Minicolumnar width, interneuronal distance, peripheral neuropil space, and compactness were evaluated in Brodmann areas 9, 21, and 22 (25, 26). Analysis by gray level index confirmed more numerous minicolumns in autism brains, and they were "narrower" with less peripheral neuropil space and increased spacing among the constituent cells as depicted in Figure 1. This architectural change, general to all areas tested, is predicted to occur during prenatal development and is postulated to reflect progressive encephalization, defined as a disproportionate increase in white matter relative to gray matter where the additional white matter primarily comprises short-range associational fibers (27). Support for this concept derives from an MRI study of subjects with autism showing a white matter volume increase in the outer subcortical white matter compartment in autism but no significant change in the inner compartment comprised of the longer fibers that communicate through the corpus callosum (28). No differences between autism and control groups were found in MRI measurements of callosal surface area, shape, or contour (29).

Finally, cortical spine densities were counted on individual cortical pyramidal cells of autistic and age- and gender-matched controls to assess postnatal culling of synaptic spines, a predictable and necessary process in normal development. Ten pyramidal cells in the superior parietal lobule (BA 7), lateral prefrontal cortex (BA 9), and middle temporal gyrus (BA 22) within 3 cortical layers (II, III, and V) were sampled from each area and visible spines counted in 25-μm-long segments of apical, basal, and oblique dendrites along the entire length of a single dendritic process (Fig. 2) (30). Results showed higher spine densities in a subgroup of individuals with ASD as compared with control subjects, and the most pronounced effect was along the midportion of apical dendrites. The most pronounced differences were seen in the temporal lobe. Contrary to the usual association of spine loss with mental retardation, increased spine density was most pronounced in the lowest functioning subgroup of cases, whereas

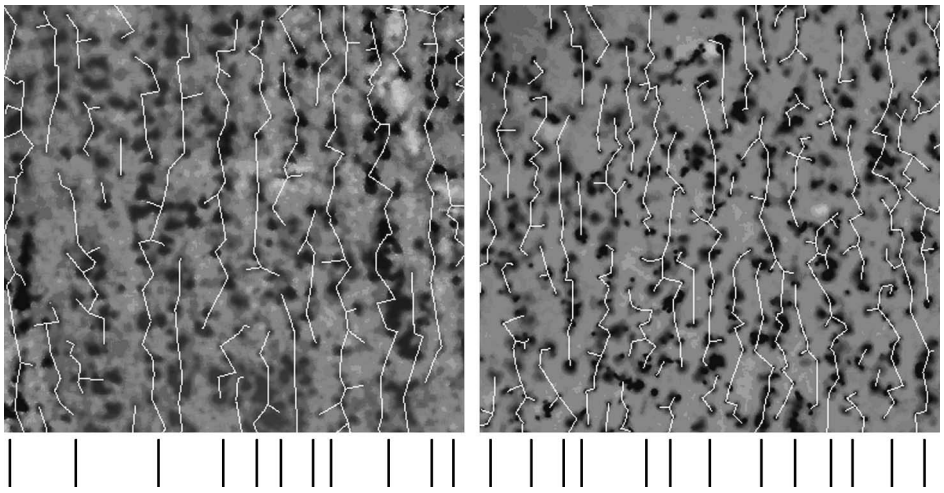

FIGURE 1. Microscopic fields (original magnification: 10×) of layer III of temporoparietal auditory area from the brain of an autistic patient **(B)** and an age-matched control **(A)**. The superimposed Euclidean minimum spanning tree indicates the cell core of the minicolumn. Lines at the bottom of each figure define the boundaries of each minicolumn showing 10 in the control brain and 12 in the brain of the autistic patient. Figure courtesy of Manuel F. Casanova, MD, University of Louisville.

ASD subjects with either mild or no mental retardation were similar to controls.

Measurements of Other Brain Regions

A volumetric and density study (31) of multiple noncortical areas in 8 autism and 8 control donors found cerebellar volume deficits in the entire cerebellum by 19% (cerebellar white matter by 30%, molecular layer by 17%, and granule cell layer by 11%). There was a 41% decrease in Purkinje cells in the autism cohort. A similar volume decrease was found in other structures of the motor system (caudate nucleus, 20%; putamen, 19%; globus pallidus; 24%; nucleus accumbens, 41%), with the total number of neurons also decreased (small and large neurons in the caudate nucleus were 22% and 29%, respectively; small and large neurons in the putamen were 24% and 51%, respectively; globus pallidus was 30%; and nucleus accumbens was 49%). In contrast to the distinct pattern of developmental abnormalities in the majority of subdivisions in the cerebellum and striatum, the volume and number of neurons in the inferior olivary and facial nerve nuclei, as well as in the dentate nucleus in the cerebellum, did not reveal significant changes. Sporadic changes were described in the memory system of brains from autism donors with numerous gaps in the pyramidal cells of Ammon's horn in the hippocampus, selective loss of stellate neurons and gliosis within the central portion of the entorhinal cortex, and ectopic clusters of neurons.

A comprehensive postmortem assessment of the amygdala complex and 5 reliably defined subdivisions, including the 1) lateral, 2) basal, 3) accessory basal, 4) central, and 5) remaining nuclei, was undertaken by Cynthia Schumann and David Amaral at the University of California Davis M.I.N.D. Institute correlating neuropathology with prior MRI findings. With antemortem MRI, they found the amygdala in autistic boys was adult size by approximately 8 years of age and did not enlarge later compared with typically developing male children who show a protracted increase in volume, reaching an adult size in late adolescence (32, 33). Patients with epilepsy were excluded from this particular study, because

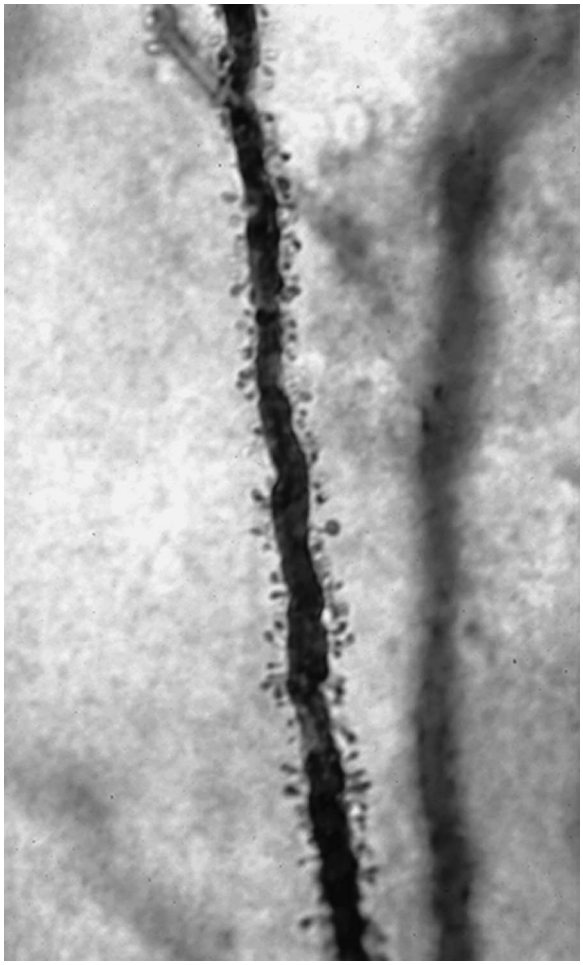

FIGURE 2. View of synaptic spines (1,000×) in human postmortem tissue using a microwave modification of the Golgi-Kopsch technique in adult, formalin-fixed, postmortem tissue. Figure courtesy of Jeffrey J. Hutsler, PhD, University of Michigan.

the latter was considered a confounding pathology. However, these seizure-associated cases will be important as a future comparison group. The subjects included 9 autism cases without seizures and 10 age-matched neurotypical controls, 10 to 44 years of age. The major finding after the initial pilot was that the entire amygdala, including its lateral nucleus, had significantly fewer neurons in autistic brains compared with age-matched controls, particularly in adults. Therefore, a picture of early enlargement and a decreased number of neurons in adulthood emerges. The authors point out that early stages of clinical depression are associated with amygdala enlargement (34), whereas long-term depression is associated with atrophy of the amygdala (35). The amygdala has also been a target of autism research because of its involvement in fear and "social" anxiety in this and other disorders such as Williams-Beuren syndrome (WBS) caused by a microdeletion of approximately 21 genes from the chromosome 7q11.23 region. WBS is a unique disorder characterized by hypersociability combined with increased nonsocial anxiety. Functional neuroimaging studies show reduced amygdalar activation for threatening *faces* in subjects with WBS but increased activation for threatening *scenes*. Activation of the orbitofrontal cortex, linking the prefrontal cortex and amygdala, was also abnormal (36).

A series of stereologic and immunohistochemical studies under the direction of Gene Blatt at Boston University expand on early findings of subcortical irregularities (37). The first is a study of 5 adult autistic and 5 age-matched controls to quantitatively analyze the parvalbumin-positive subpopulation of GABAergic interneurons in the dentate gyrus, hippocampus proper, prosubicular, and subicular subfields to see if these interneurons show increased packing density similar to that reported for neurons in general within the pyramidal layers of the hippocampus and subiculum. Results revealed a statistically significant increase in cell packing density of PV-positive interneurons in the CA1 and CA3 regions of autistic brains (38).

A trio of studies investigated the functional and temporal relationships between the reduction of Purkinje cells in the cerebellum and cytoarchitectonic irregularities in the synaptically related principle olive (PO). In the first study, the total number of neurons and neuronal size were assessed in the PO of the inferior olivary complex from 5 adult male autistic and 5 control brainstems cut in 50-μm-thick serial sections with one series stained with cresyl-echt violet. Results showed no significant differences in total neuronal number or size in the autistic brains when compared with controls (the mean number of PO neurons in the autistic brains was 661,000 and in controls was 691,000, and the mean surface area of neurons in the autistic brains was 1679.2 μm^2 and in controls was 1644.4 μm^2) (39). In a second experiment, the numbers of basket cells and stellate cells were counted in relation to Purkinje cells in the 6 autistic and 4 control cerebellar sections using immunostaining for parvalbumin. The result was a normal number and density of basket cells and stellate cells in all 6 autism cases despite a reduced number of PCs in 3 cases (40). Last, using peripherin as a marker for climbing fibers, the density and distribution of the olivocellebellar projection was measured in 6 adult autistic and 6 age-matched control brains, as well as density in the white matter near the dentate nucleus. The innervation of Purkinje cells by olivocerebellar climbing fibers was similar in the autistic and control brains, and both had an extensive branching pattern around the primary dendrites as well as to neighboring Purkinje cells. Peripherin-positive fibers were found to innervate the dentate nucleus in both groups; however, the fibers in the autism cases were disorganized and appeared less dense (41).

The tentative conclusion from this series of pilot studies is that the estimated timing of Purkinje cell disappearance, in those cases where it does occur, would be some time after 32 weeks of gestation. The authors suggest this as the only timeframe that would allow for Purkinje neurogenesis, migration, innervation by the climbing fibers, and successive envelopment by basket cells to take place, forming the "basket." The window of time for disappearance of Purkinje cells would extend from 32 weeks to birth based on the observation that the decreased number of Purkinje cells in the cerebellum is probably not associated with loss of olivary neurons (after birth, Purkinje cell loss is accompanied by a loss of PO neurons). This (prenatal) timeframe also suggests autolysis from agonal (postnatal) states may not be confounding the observed reduction in Purkinje cells or the disruption of olive projections to the dentate.

Neurotransmitter Systems/Synaptic Processes

The hippocampus, cortex, and cerebellum have been evaluated for changes in various neurotransmitter systems. A study surveying 4 neurotransmitters systems is highlighted as well as more specific investigations of the serotonergic, cholinergic, and GABAergic systems.

A study in 2001 of 8 types of neurotransmitter receptors from 4 systems (GABAergic, serotonergic, cholinergic, and glutamatergic) in the hippocampus showed a specific and substantial reduction in the GABAergic system using 3[H]-flunitrazepam-labeled benzodiazepine-binding sites and 3[H]-muscimol-labeled GABA(A) receptors in autism cases (42). This was in contrast to no change in the density and distribution of 6 other receptors studied (3[H]-80H-DPAT-labeled 5-HT1A receptors, 3[H]-ketanserin-labeled 5-HT2 receptors, 3[H]-pirenzepine-labeled M1 receptors, 3[H]-hemicholinium-labeled high-affinity choline uptake sites, 3[H]-MK801-labeled NMDA receptors, and 3[H]-kainate-labeled kainate receptors).

Technologic advances in recent years allow better discrimination of the components of neurotransmitter systems and the serotonergic system is especially interesting. For example, Blatt's group measured 5-HT receptor density and anatomic distribution (5HT2a and 5HT1a receptor subtypes [5HTR1a, 5HTR2a] and the 5HT uptake site [5HTU]) in the anterior cingulate cortex and Brodmann area 24 in 7 adult autistic and 10 age-matched control brains. They found decreases in the density of 5HTRla or 5HTR2a, but no significant differences in the density of the 5HTU site (43). The cingulate is involved in higher-order integrative behaviors and implicated in autism-associated limbic pathology, as shown by specialized imaging techniques. For instance, in adult subjects with Asperger's, in vivo single photon emission tomography reveals a significant reduction in cortical 5HT2a receptor binding in the whole cingulate cortex (as well as the bilateral

front and superior temporal cortex and the left parietal cortex). Reduced receptor binding has been associated with abnormal social communication (44).

Serotonin's role in autism derives from early reports of blood platelet hyperserotonemia and the general efficacy of selective serotonic reuptake inhibitors to assuage obsessive/compulsive and repetitive behaviors. A study of platelet 5-HT levels in Dutch children and young adults, recruited from 2001 through 2003, with an ASD (autism, Asperger's, and PDD-not otherwise specified [PDD-NOS]; n = 81) or with mental retardation (n = 54) but without PDD, and in normal controls (n = 60) showed a bimodal distribution in platelet 5-HT values in the ASD group (45). Although evaluation of the hyperserotonemic subgroup's behavioral variables did not show segregation of any specific autistic disorder with platelet 5-HT levels, interest continues in this measure as an ASD biomarker.

Cholinergic systems have similarly been analyzed in autistic brain tissue. In the cerebellum, nicotinic receptor binding the agonist, epibatidine was significantly reduced by 40% to 50% in the granule cell, Purkinje, and molecular layers in the autistic compared with the normal group ($p < 0.05$). There was an opposite increase (3-fold) in the nicotinic receptor binding alpha-bungarotoxin (to the alpha7 subunit), which reached statistical significance in comparisons from the internal granular cell layer ($p < 0.05$). These receptor changes were accompanied by significant reductions ($p < 0.05$) and nonsignificant increases, respectively, of alpha4 and alpha7 receptor subunit expression levels by Western blot analysis (46). A substantial depletion of MAP2-reactive neurons in the dorsolateral prefrontal cortex was reported by the same group of investigators (47).

GABA synthesis in the cerebellum and parietal cortex, as measured by glutamic acid decarboxylase (GAD), was found to be significantly reduced (48). This system is of particular interest because the reelin gene (*RELN*) is an autism candidate gene from family linkage studies (49). The reelin glycoprotein is a secretory serine protease and has dual roles in the mammalian brain. During embryonic development, it guides neurons and radial glial cells to their correct positions; and in the adult brain, reelin is considered a signaling protein underlying synaptic plasticity (50). A follow-up study using SDS-PAGE and Western blotting of Reelin protein was done on postmortem superior frontal, parietal, and cerebellar cortices of age, gender, and postmortem interval-matched autistic and control donor brains. Quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of Reelin, VLDL-R, Dab-1, and GSK3 mRNA species in superior frontal and cerebellar cortices of autistic and control subjects was also performed, and the result was that Reelin 410, 330, and 180 kDa values were reduced significantly in frontal and cerebellar and nonsignificantly in parietal areas of autistic brains versus control subjects, respectively. Reelin and Dab-1 mRNA were significantly reduced, whereas the mRNA for the Reelin receptor, VLDL-R, was significantly elevated in superior frontal and cerebellar areas of autistic brains versus control brains, respectively (51). The concomitant reduction in the Reelin protein and increase in Reelin receptor mRNA point to impairments in the Reelin signaling system in autism and might account for some of the structural central nervous system and cognitive deficits observed in this disorder. Other potential molecular mechanisms are reviewed in the following section.

Molecular Mechanisms

Several high-throughput microarray analyses have been performed to globally evaluate gene expression in various brain regions; the one published to date shows numerous up- and downregulated genes in an analysis of 10 autism and 23 matched control brains (52). The mRNA levels of 2 components of the glutamate system, excitatory amino acid transporter 1 and glutamate receptor AMPA 1, were significantly increased in autism tissue. When AMPA- and NMDA-type glutamate receptor densities were examined with receptor autoradiography in the cerebellum, caudate–putamen, and prefrontal cortex, AMPA-type glutamate receptor density was decreased in the cerebellum of individuals with autism ($p < 0.05$), suggesting that AMPA-type glutamate receptors and glutamate transporters may contribute to the pathogenesis of this disorder.

The Angelman gene (*UBE3A*), which encodes the E6-AP ubiquitin ligase, is another candidate autism gene and resides in the 15q11-q13 region. Autism brain tissue showed abnormal DNA methylation at the 5′-CpG island of *UBE3A* and decreased E6-AP protein in one of 17 autism brains. The research team suggests a model called MEGDI (mixed epigenetic and genetic and mixed de novo and inherited) for autism with both de novo and inherited contributions that are brain-specific and would not be detectable using DNA from blood or cultured cells (5).

The Angelman gene *UBE3A* product, E6-AB, has been assessed in brain tissue in relation to the function of the Rett Syndrome gene, *MECP2*. This X-linked-dominant disorder, caused by MECP2 mutations, has phenotypic and genetic overlap with autism. The *MECP2* gene encodes the methyl-CpG-binding protein 2 that acts as a transcriptional repressor. Multiple quantitative methods (automated quantitation of immunofluorescence and in situ hybridization by laser scanning cytometry on tissue microarrays, immunoblot, and TaqMan PCR) were used to test the hypothesis that a MeCP2 deficiency may affect the expression levels of both E6-AB and the product of a neighboring autism candidate gene, *GABRB3,* on chromosome 15q11-q13 (53). Significant reductions in E6-AP expression were found in 2 different Mecp2-deficient mouse strains and human Rett, Angelman, and autism brains compared with controls. The nonimprinted gene from 15q11-q13, *GABRB3*, encoding the beta3 subunit of the GABA(A) receptor also showed significantly reduced expression in multiple Rett, Angelman, and autism brain samples, suggesting gene dysregulation within 15q11-q13 in Rett, Angelman, and autism and implicating MeCP2 in the regulation of *UBE3A* and *GABRB3* expression in postnatal mammalian brains.

Histone methylation is another epigenetic mechanism that governs long-term eukaryotic translational regulation. Histones and their covalent modifications (such as methylation and acetylation) are epigenetic regulators of chromatin structure and gene expression. Notably, histone immunoreactivity is maintained at robust levels in postmortem brain tissue (Fig. 3). Recently, Akbarian and colleagues showed that developmental

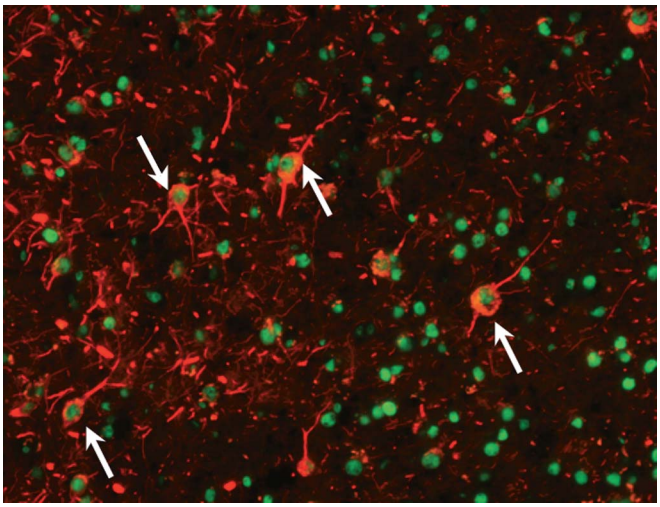

FIGURE 3. Histone immunohistochemistry in human postmortem cerebral cortex. Horizontal section showing large neurofilament H immunoreactive pyramidal neurons in red (arrows) and robust immunoreactivity for H4-Acetyl-K8 in nuclei (green). Autolysis time: 16.5 hours. Magnification: 20×. Figure courtesy of Schahram Akbarian, MD, PhD, University of Massachusetts Medical School & Brudnick Neuropsychiatric Research Institute.

regulation of glutamate receptor expression in human brain is associated with histone methylation changes at gene promoters (54) and an association between selected histone modifications and downregulated metabolic gene expression in the frontal lobe of schizophrenics. Presently, the team is conducting a comprehensive case–control study on histone methylation patterns in the frontal lobe of subjects diagnosed with autism spectrum disorder or schizophrenia. These studies include GAD1(GAD67), a GABA-regulating gene that confers genetic risk for childhood-onset schizophrenia.

A proteomic approach to identify protein abnormalities in autopsied autism brains has identified, in 4 of 8 autism brains, an increase in polarity (more acidic) of glyoxalase I (Glo1) by 2-dimensional gel electrophoresis. Sequencing of the *GLO1* gene identified a single nucleotide polymorphism, C419A, that causes an Ala111Glu change in the corresponding protein sequence. Population genetics studies of the *GLO1* C419A single-nucleotide polymorphism in autism (71 samples) and normal neurologic controls (49 samples) showed a significantly higher frequency of the A419 allele (frequency 0.6 in autism and 0.4 in controls, one-tailed Fisher test, $p < 0.0079$). Biochemical measurements in autism brains have revealed a 38% decrease in Glo1 enzyme activity in autism brains, and Western blot analyses have also shown accumulation of advanced glycation end products (55).

Neuroimmunology

Autoimmunity is another area of convergent interest in autism. A recent report of plasma antibody reactions with brain extracts showed, by quantitative immunoblotting, that a putative isoform of myelin basic protein discriminated between autistic patients and controls (56). Evidence for the presence of an active and ongoing neuroinflammatory process in the cerebral cortex, white matter, and notably in the cerebellum of postmortem brains or cerebral spinal fluid (CSF) of autistic patients was obtained using various techniques (immunocytochemistry, cytokine protein arrays, and enzyme-linked immunosorbent assays [ELISA]) (57). Fresh-frozen tissues available from 7 patients and cerebrospinal fluid (CSF) from 6 living autistic patients were used for cytokine protein profiling. These immunocytochemical studies showed marked activation of microglia and astroglia and cytokine profiling showed that of many possible targets, macrophage chemoattractant protein (MCP)-1, and tumor growth factor-1 were the most prevalent cytokines in brain tissues. This suggests an active neuroinflammatory process in the cerebral cortex, white matter, and cerebellum of autistic patients. The CSF showed a unique proinflammatory profile of cytokines, including a marked increase in MCP-1. Another indication of age-related immune activity results from an investigation of sections from Wernicke's area and the gyrus angularis from brains of deceased autistic patients (17–45 years old) and control brains (14–46 years old) that were either Nissl-stained for morphometric cytoarchitectonic analyses or examined for lipofuscin autofluorescence (58). The neuronal density in normal subjects gradually decreased with age, whereas the number of glial cells increased. In contrast, this group reported a decrease in cortical neurons in the youngest autistic patients, and these young patients also had conspicuous gliosis across all layers. The number of neurons showing lipofuscin intracytoplasmic deposits increased with age both in controls and autistic patients, but the latter had significantly more such cells at all ages studied. GFAP immunoreactivity, indicative of active astrocytes, was increased in the autism cases as well.

CURRENT RESEARCH STRATEGIES

Brain tissue studies can clearly play an important role in defining the processes involved in autism; however, the next phase of neuropathologic research in this field requires collaborative planning, with clear translational aims, an evidence-based approach with integration of data from various projects. The resource, donated brain tissue, is often the rate-limiting factor in moving from speculative findings to a real understanding of mechanisms that will allow for detection or treatment of autism spectrum disorders.

Improving the resource is the goal of the Autism Tissue Program (ATP)—The Gift of Hope—established in 1998 by the Autism Society of America (ASA) Foundation and the National Alliance for Autism Research (NAAR). The ATP web site, www.memoriesofhope.org, lists current projects and research progress (abstracts and publications) and supports online donor registration. The ATP national outreach/education program encourages brain donation to the National Institutes of Health (NIH)-designated autism brain bank, the Harvard Brain Tissue Resource Center (HBTRC). After next of kin consent, the HBTRC staff arranges the recovery of the brain and oversees shipping details. Funds from a grant supplement to HBTRC reimburse tissue recovery costs and basic programmatic operations while NAAR continues to provide additional fiscal and administrative support.

The Children's Health Act of 2000 (Public Law 106-310) provided an important federal mandate for a program to collect and share tissue samples for autism research. The Act also directed NIH to establish autism centers around the United States to link clinical and biomedical research. These centers share a common research database, and postmortem research will clearly benefit from consolidated donor information associated with MRI, positron emission tomography, electroencephalography, family genetics, biochemistry, and physiological studies.

By the end of 2004, the ATP had acquired and banked brain tissue specimens from 66 autism spectrum donors, 5 first-degree relatives, and 7 grandparents. A Tissue Advisory Board (TAB), comprised of experts in neuroimmunocytology, neuropathology, molecular genetics, neuroimaging, neurology, and clinical diagnostics, but who do not themselves conduct studies of autism tissue, recommends tissue acquisition and storage protocols, reviews tissue requests, and approves tissue distribution. ATP tissue applicants are required to sign a data-sharing agreement and have received support to participate in meetings to discuss progress. To date, brain tissue specimens have been distributed to 42 investigators and as many as 19 different laboratories received tissue from one of the specimens.

Several tissue-sharing projects have resulted from the priority set by the TAB to expand the utilization of this rare resource to accommodate more investigations. For example, 50- and 100-μm-thick sections of the limbic region blocked for an amygdala study (59) are stored cryoprotected for redistribution to approved studies (Institutional Review Board approval and tissue transfer agreements are monitored by the ATP). A tissue microarray with 99 samples of various regions of autism brains, control brains, and control nonneural tissue was constructed at the Johns Hopkins Tissue Array Laboratory (60). Figure 4 shows stained sections cut from the array. Over 150 sections can be cut from one array and distributed to multiple investigators for various staining and receptor-binding experiments. Banked cDNA, RNA, and protein samples generated by various investigators can also be shared with other scientists. Likewise, a collaborative project generated a "third" series of 200-μm celloidin-embedded hemispheric coronal sections from 10 autism and 10 control subjects. Termed the ATP Brain Atlas Project, a collaboration between Jerzy Wegiel at the Institute for Basic Research in New York and Christoph Schmitz at the University of Maastricht, The Netherlands, this series is reserved for future studies to complement comprehensive stereologic studies from the first 2 series (23, 31). An additional 12 autism hemispheres were added to the Project in mid 2005; approximately one third of these cases have frozen sections from the contralateral hemisphere for genetic studies. The aim is to detect patterns of developmental abnormalities in the brains of people with autism, integrate quantitative measures of developmental abnormalities with mathematical models of progression of age-related changes, and correlate morphometric measures of developmental abnormalities with clinical features of autism.

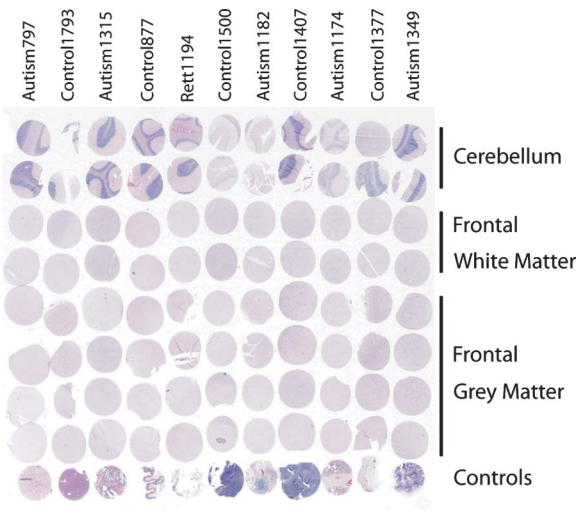

FIGURE 4. Array made by Charles Eberhart at the Johns Hopkins Tissue Microarray Core Facility from cerebellar and frontal cortex tissues from the University of Maryland NICHD Brain Bank. This hematoxylin & eosin-stained slide shows a section cut from an array made of 1.5-mm punches from ''superficial'' and ''deep'' frontal cortex, along with subcortical white matter, from the same block and cerebellar cortex from a second block. Control cases alternate with affected cases in columns and the bottom row contains reference nonneural tissue: from left to right: kidney, liver, prostrate, skin, lung, tonsil, bladder, thymus, placenta, gallbladder, and salivary gland. Figure courtesy of Charles G. Eberhart, MD, PhD, Johns Hopkins University School of Medicine.

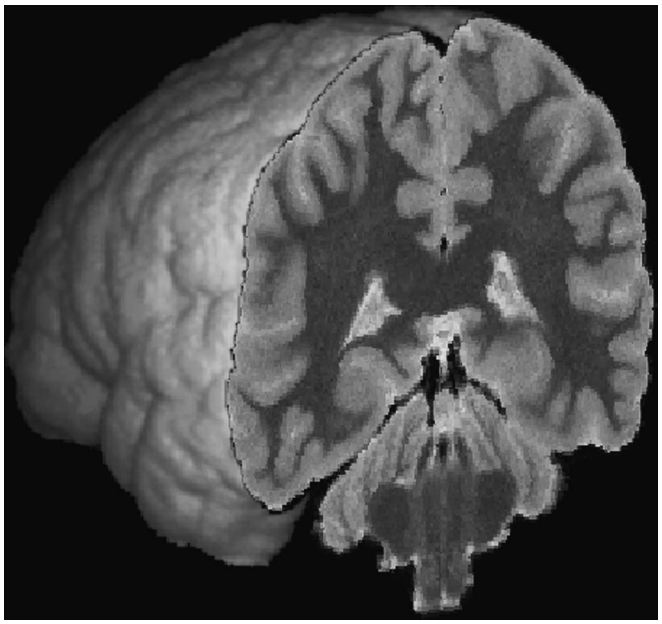

FIGURE 5. Three-dimensional reconstructed image of whole brain; tissue is fixed a minimum of 6 weeks in formalin and scanned, placed in a humidity-controlled chamber on a plexiglas support, and scanned at 1.5 Tesla using fast spin echo. Analyze 6.1 (Mayo Foundation) was used to import, preprocess, and align the brain used for this image. Software mri3dX 5.0 (Aston University, UK) was used to render the final 3-dimensional image. Figure courtesy of Cynthia Schumann, PhD, UC Davis, California.

Researchers cataloging differences between the autistic and nonautistic brains are using sophisticated tools that they have perfected for tissue analysis. These include direct visual measurements, digitized stained hemibrain sections, stereologic measures of cells and dendritic structures in various brain regions, and postmortem MRI as seen in Figure 5 (methods detailed in [59]). Several have developed stereologic tools such as cortical "nearest neighbor" cell analyses to identify changes that relate to neurodevelopment (61), gray-level indexing to measure cortical minicolumns architecture (25), and 3-dimensional reconstructions of stained serial sections (62).

New technology developed to measure the expression of microRNA also provides an opportunity to study post-transcriptional modifications that potentially affect developmental timing, neuronal differentiation, cell proliferation, and programmed cell death (63). A new technique called the RNA-primed, array-based Klenow enzyme (RAKE) assay offers unique advantages for specificity over Northern blots or other microarray-based expression profiling platforms and is especially important for use on archived, formalin-fixed, paraffin-embedded tissue (64).

Three studies are underway using tissue samples from a set of autism and control donor brains. A collaborative multi-institutional project in the United States and France is sharing samples from the HBTRC and NICHD neurodevelopmental brain bank at the University of Maryland. The first is assessing the mRNA and protein levels of vesicular glutamate transporter proteins (VGLUTs) using new specific markers. The *VGLUT* genes are differentially expressed with respect to both brain region and brain development as determined by staining for the transporters. VGLUT 1 is present in excitatory neurons in the cerebral and cerebellar cortex as well as in the hippocampus, VGLUT2 is expressed by glutaminergic neurons in the embryonic fore- and hindbrain (diencephalon and rhombencephalon), and VGLUT3 is found in cholinergic interneurons of the striatum and in serotonergic neurons of the raphe nuclei (65). The second explores expression of *ENGRAILED* 2 (*EN2*), localized to 1q22, with a very large linkage score for schizophrenia and autism and implications for regulation of cerebellar development and a second candidate gene located in a broader area of this chromosome arm in region (1q22-23)—*CAPON*, carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase (66). The third focuses on candidate genes on chromosomes 2 and 7. These are the *SLC24A12* gene, located on 2q24-33, that contributes to modifications of dendritic spines in mice (67), and the *AUTS2* gene on chromosome 7, noted for a translocation breakpoint found in a pair of twins with autism and postulated to be functionally linked to *RELN* (68, 69). This last study uses laser capture technology to pluck single cells from stained tissue to obtain a homogeneous population of cells as seen in Figure 6.

The scarcity of frozen tissue is a consistent concern. The choice of tissue processing protocols depends on a variety of factors that include postmortem interval, local tissue recovery capability, and circumstances of the death. When possible, the whole fresh brain is recovered and shipped by courier to the bank for processing (half formalin-fixed; half snap-frozen). Often, the remote location or the sudden and unexpected nature of the deaths of those with autism make it difficult to obtain timely consent, resulting in prolonged postmortem interval and either a "half and half" preparation onsite (a formalin-fixed and a frozen whole hemisphere) or a whole formalin-fixed brain. Additionally, the ATP relies heavily on cooperation from medical examiners whose forensic work determines the release of consented tissue. The HBTRC will

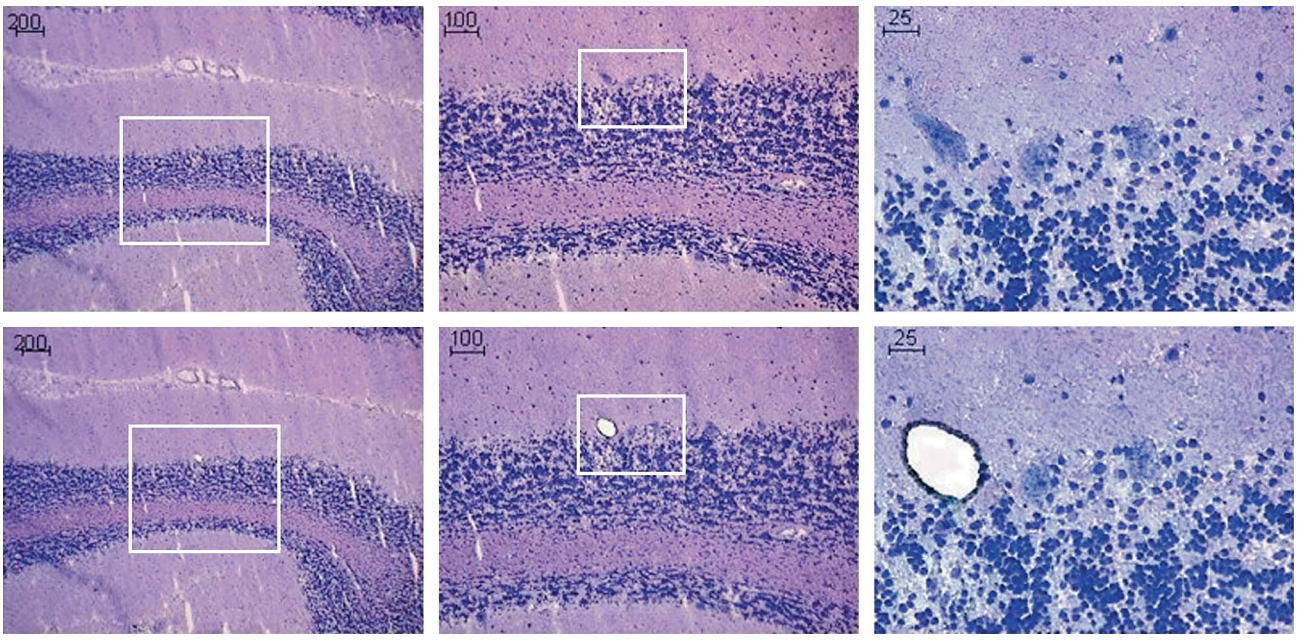

FIGURE 6. Purkinje cell laser microdissection from human cerebellum tissue. Figure courtesy of Michel Simonneau, MD, PhD, INSERM U675, Paris.

provide a consultation and written neuropathology report within 30 days. The consent form and tissue processing protocols are published on the HBTRC site (http://www.brainbank.mclean.org).

A major challenge for behavioral neuropathology is to link the nature of brain pathology with the functional deficits that define a disorder. Success in interpreting data depends on both ante- and postmortem characterization of donors and brain tissue. Specific donor variables may provide important clues; for instance, a donor with autism also had neurodegeneration with brain iron accumulation (NBIA). This case provides a rare opportunity to study both disorders and, more specifically, to study oxidative stress in autism—an area of interest based on findings of lipid peroxidation and reduced serum levels of transferrin (iron-binding protein) and ceruloplasmin (copper-binding protein) in serum of autistic children compared with their developmentally normal nonautistic siblings (70).

Thus, special attention is paid to the donor's medical history, demographic attributes (age, gender, ethnicity), comorbid medical conditions (e.g. septal optic dysplasia, colombata, syndactyly, epilepsy), and factors such as agonal state, tissue fixation, and postmortem interval. An ATP clinician visits donor families for diagnostic assessment and to obtain records. This data is loaded into the ATP Portal, a dynamic, personalized data access system based on Oracle technology (www.atpportal.org). It provides access to donor information and permits sorting of existing cases based on donor antemortem history (e.g. medical documentation includes immunization, medication, hospitalizations, and seizure history) as well as agonal state and postmortem tissue conditions. Data on donor brains can be extracted from the ATP Portal and used for simple group comparisons; for example, autism and control fresh brain weights show a normal distribution for each group with a trend toward heavier brains in the autism group (Fig. 7). There is an administrative function to track donor registrants, tissue requests from investigators, and tissues awarded. There is also an ongoing assimilation of datasets derived from research on the core group of donors for neuroinformatics analysis by investigators.

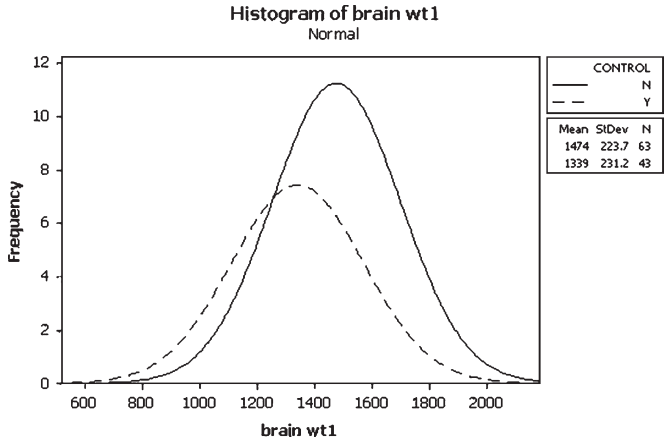

FIGURE 7. Distribution of fresh brain weight for ATP control (dashed line) and autism (solid line) donor cases. Both sexes are included. Figure courtesy of Michael Brimacombe, PhD, University of Medicine and Dentistry of New Jersey (UMDNJ).

SUMMARY

Autism neuropathologic investigation continues to grow and benefit from a collaborative approach by both federal institutions and advocate organizations. Groups are emerging to explore how histology data might relate to brain circuitry and how to unravel the genetic/metabolic cascade unleashed during neurodevelopment. However, barriers to research remain. Variability within the autism group is a common finding in most of the studies. Additional cases and analyses of the contributions of age, agonal state, postmortem interval, comorbid conditions such as epilepsy, levels of cognitive functioning, and morphometric features are required. Larger numbers of well-characterized brain specimens are needed to transform these pilot projects with provocative preliminary findings about anatomic and molecular changes to studies with adequate statistical power for definitive conclusions.

At present, there is a general lack of matched control tissues in the 2- to 15-year-old age group. This problem is so significant that several projects with brains from autistic children cannot be completed until such tissue becomes available. Pediatric brain donation presents obstacles not experienced with geriatric donation programs; there is often no indication of impending death and donation depends on the medical examiner's willingness to provide tissue for research. Therefore, the ATP is working with organ and tissue banks that, in conjunction with trained hospital staff, approach families for consent to donate within 2 to 3 hours of death.

Neuropathology and experimental neurology professionals are invited to join this research effort by advocating for brain donation when possible, continuing to set high standards for tissue recovery and mounting tissue-based investigations.

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