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14. ABSTRACT
The postmortem study of the historically largest cohort including 32 brains of individuals with idiopathic autism, 12 brains of individuals with autism associated with chromosome15 duplication and 28 brains of control subjects, and examination of 36 brain cytoarchitectonic subdivisions was the first attempt to define a global pattern of developmental changes in autism. The study revealed defects of neuronal migration, proliferation and dysplastic changes as a major contributor to seizures. Examination of 2 to 64 year old subjects revealed the first model of brain region-specific modifications of neuron growth trajectories during the lifespan demonstrating desynchronized development of interacting neurons. Neuron soma volume was significantly smaller in all examined regions in 4-8 year old children. Significant increase of neuron size in teenagers and adults to and in some regions above control level without equally significant clinical improvement suggests that delayed growth does not reproduce normal neuronal growth, connectivity and function. Limited alterations in volume of a few structures and number of neurons indicate that alterations of neuronal growth are a major contributor to the clinical autism phenotype.

15. SUBJECT TERMS- Autism neuropathology, chromosome 15 duplication, brain, neuronal volume, neuronal density, total number of neurons, trajectories of neuronal growth, global pattern of brain pathology, desynchronized neuronal growth, clinico-pathological correlations,

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FINAL REPORT
2013

Program Project Title: Characterization of the pathological and biochemical markers that correlate to the clinical features of autism

Program Project PI: Jerzy Wegiel, Ph.D.; Co-PI: W. Ted Brown, M.D., Ph.D.

The overall aim of this multidisciplinary program project is to establish correlations between morphological and biochemical markers of autism and the clinical symptoms of the disorder.

Start Date: 10-01-2008 End Date: 09-30-2-11

No cost extension to: 04-21-2013

SUBPROJECT 2

Contribution of significant delay of neuronal development and metabolic shift of neurons to clinical phenotype of autism

Subproject 2 P.I.: Jerzy Wegiel, Ph.D.

INTRODUCTION

The overall aim of this multidisciplinary program project was to establish correlations between morphological and biochemical markers of autism and clinical symptoms of the disease. To achieve these goals, we proposed three subprojects. The factor integrating these three subprojects and three closely collaborating groups is the concentration of a broad spectrum of aims on the historically largest cohort examined postmortem in one project. The project created a unique opportunity to detect the patterns of global brain pathology and trajectories of changes during the entire lifespan of autistic individuals.

This Program Project is focused on the detection of:

(a) mechanisms leading to morphological changes and the clinical autism phenotype,
(b) morphological, morphometric and biochemical markers of autism,
(c) correlations between type, topography and severity of structural and biochemical alterations and clinical manifestations of autism, and
(d) those pathological alterations that might be a target for treatment.

Project expansions. Research was performed consistently with the original Program Project and Subproject 2 aims and timetable. However, material preserved created an opportunity for expansion of research by (a) adding approximately 30% more brains, (b) adding 50% more brain cytoarchitectonic subdivisions, (c) redirecting research from a localized models to the first global model of developmental alterations in autism, (c) adding new subprojects such as the study of the role of floccular dysplasia in altered gaze control and (d) exploration of the feasibility of a study of the serotonergic system in autism.

Material : We examined the brains of 72 subjects including: 32 brains of autistic people, 12 brains of individuals with autism associated with chromosome15 duplication (dup15) and 28 brains of control subjects, exceeding the original plan by approximately 30%.

The neuropathological criteria were established in cooperation with Project 1. Cases not meeting the ADI-R criteria and cases with signs of comorbidity, as well as with premortem and postmortem changes affecting brain structure were excluded from the morphometric studies.

Brain structures selected for morphometric study of developing, mature and aging brain of autistic people.
Consistently with the Statement of Work, we examined 10 brain subregions, most likely affected by developmental delay and metabolic aberration in adults and aged people with autism and pathology linked with clinical symptoms of autism:
(1) Amygdala involved in social interactions, anxiety, fear, aggression, memory and cognition).
(2) Caudate nucleus, (3) putamen, (4) globus pallidus (involved in stereotypes and rituals).
(5) N. accumbens ("social brain", reward system).
(6) Nucleus supraopticus and (7) N. paraventricullaris. These two hypothalamic nuclei are (a) the source of numerous growth and trophic factors necessary for normal brain development and function, and (b) the source of factors regulating social memory and attachments, emotional responses, and cognitive functions.
(8) Cerebellum: cortex, white matter (gaze control, language, pointing, motor functions, cognition).
(9) Dentate nucleus (language, pointing, motor functions, cognition).

Project expansions: To achieve a more global view of the pattern of brain developmental alterations we expanded the list of examined brain regions by adding eight brain structures, and their 10 anatomical subdivisions (layers and sectors):
(1) Entorhinal cortex (input to the memory system)
(2) Hippocampal formation (memory system; processing and storage)
(3) Thalamus (a key component of networks implicated in attention, memory, language, and emotional processing)
(4) Claustrum (integration of function of several brain modalities contributing to cognition)
(5) Magnocellular basal complex (including nuclei providing acetylcholine for the entire brain)
(5) Substantia nigra (source of dopamine controlling motor, reward and other systems)
(6) Inferior olive (part of the olivo-floccular system controlling eye movement)
(7) Nucleus of facial nerve (facial expression)
(8) Cerebellar flocculus. Due to a specific role of the cerebellar flocculus in gaze control, we designed a detailed study of the unique developmental alterations in the flocculus.
(9) Raphe nuclei in the brainstem (the only source of brain serotonin acting both as neurotransmitter and neurotrophic factor).

After these expansions, the study of a global pattern of brain developmental abnormalities doubled the number of examined regions/structures to 18 localized models of developmental alterations in the brain of autistic and control subjects.

Examination of 22 subdivisions of these structures/regions increased the total number of structure and subregions examined to 40. List of subregions includes:

(1-4) four nuclei in the amygdala including lateral, basal, accessory basal and central
(1-5) nucleus;
(5-6) two subdivisions in the globus pallidus (external and internal globus pallidus);
(7-10) four layers in the entorhinal cortex (islands, layer III, V, and VI);
(11-14) four sectors in the cornu Ammonis (sector 1, 2, 3, and 4);
(15-16) two parts in the claustrum (prepiriform claustrum and compact or insular claustrum);
(15-19) four subdivisions of the magnocellular basal complex (CH1, 2, 3, 4);
(20-22) Three major subdivisions of the substantia nigra (dorsal and ventral tier, and lateral nucleus);

Outcome:
(a) Examination of the entire brain hemispheres in the historically largest cohort of 72 subjects increased the power of established global patterns of developmental alterations in autism.
(b) Examination of 18 brain subregions/structures and their 22 subdivisions results in the most complex study of 40 brain subdivisions in the largest cohort of subjects diagnosed with idiopathic autism (autism with unknown etiology), autism with known etiology (dup15q11.2-q13), and age-matched controls.

Therefore, this study provides new information about:
(a) Interindividual differences;
(b) Global model of the trajectory of neuron growth throughout the lifespan in idiopathic autism, autism associated with dup(15) and in control cohort;
(c) Region-specific differences of abnormal neuron growth;
(d) Desynchronized neuron growth in different brain regions/structures;
(e) Number of neurons in examined brain structures and subdivisions (unbiased morphometric study revealed only marginal modifications of the numerical density and total number of neurons per subdivision).
(f) Volume of examined brain regions/structures (there is no evidence of significant alterations of the volume of 18 major brain subdivisions).

BODY

Summary of 11 subprojects closing Project 2

A. Contribution of focal developmental alterations to clinical symptoms of autism

1. **Type, topography and sequelae of neuropathological changes shaping clinical phenotype of autism.** Wegiel J, Wisniewski T, Chauhan A, Chauhan V, Kuchna I, Nowicki K, Imaki H, Wegiel J, Ma SY, Wierzba-Bobrowicz T, Cohen IL, London E, Brown WT. In: Autism: Oxidative Stress, Inflammation, and Immune Abnormalities. Ed.: Abha Chauhan, Ved Chauhan and W. Ted Brown. Taylor & Francis/CRC Press, Boca Raton, FL, 2010, pp. 1-34.

2. **The neuropathology of autism: defects of neurogenesis and neuronal migration, and dysplastic changes.** Wegiel J, Kuchna I, Nowicki K, Imaki H, Wegiel J, Marchi E, Ma SY, Chauhan A, Chauhan V, Wierzba Bobrowicz T, de Leon M, Saint Louis LA, Cohen IL, London E, Brown WT, Wisniewski T. Acta Neuropathol, 2010, 119,755-770.

The aim of this study was to detect the patterns of focal qualitative developmental defects and to identify brain regions that are prone to developmental alterations in autism. Formalin-fixed brain hemispheres of 13 autistic (4–60 years of age) and 14 age-matched control subjects were embedded in celloidin and cut into 200-μm-thick coronal sections, which were stained with cresyl violet and used for neuropathological evaluation. Thickening of the subependymal cell layer in two brains and subependymal nodular dysplasia in one brain, are indicative of active neurogenesis in two autistic children. Subcortical, periventricular, hippocampal and cerebellar heterotopias detected in the brains of four autistic subjects (31%) reflect abnormal neuronal migration. Multifocal cerebral dysplasia resulted in local distortion of the cytoarchitecture of the neocortex in four brains (31%), of the entorhinal cortex in two brains (15%), of the cornu Ammonis in four brains and of the dentate gyrus in two brains. Cerebellar floculonodular dysplasia detected in six subjects (46%), focal dysplasia in the vermis in one case, and hypoplasia in one subject indicate local failure of cerebellar development in 62% of autistic subjects. Detection of floculonodular dysplasia in only one control subject and of a broad spectrum of focal qualitative neuropathological developmental changes in 12 of 13 examined brains of autistic subjects (92%) reflects multiregional dysregulation of neurogenesis, neuronal migration and maturation in autism, which may contribute to the heterogeneity of the clinical phenotype.

3. Differences Between the Pattern of Developmental Abnormalities in Autism Associated with Duplications 15q11.2-q13 and Idiopathic Autism

Wegiel J, Schanen CN, Cook EH, Sigman M, Brown WT, Kuchna I, Nowicki K, Wegiel J, Imaki H, Ma SY, Marchi E, Wierzba-Bobrowicz T, Chauhan A, Chauhan V, Cohen IL, London E, Flory M, Lach B, Wisniewski T. J Neuropathol Exp Neurol 2012, 71, 382-397.

See Report #1 (Dr. Thomas Wisniewski PI)

4. Contribution of olivo-floccular circuitry developmental defects to atypical gaze in autism

Wegiel J, Kuchna I, Nowicki K, Imaki H, Wegiel J, Ma SY, Azmitia EC, Banerjee P, Chauhan A, Chauhan W, Cohen IL, London E, Brown WT, Wisniewski T. Brain Res 2013,1512:106-122.

Individuals with autism demonstrate atypical gaze, deficits in facial perception, altered movement perception, and impairments in smooth pursuit (Rosenhall et al 1988; Scharre and Creedon, 1992; Takarae et al 2004 ab). A substantial number of Purkinje cells in the cerebellar flocculus receive converging visual inputs from functionally distinct portions of the retina and subserve the neural mechanisms for oculomotor control during slow eye movements.

The flocculus provides the oculomotor system with eye position information during fixation and with eye velocity information during smooth pursuit (Noda and Suzuki 1979). Our studies indicate that in majority of autistic subjects the flocculus is affected by dysplastic changes (Wegiel et al 2010).

The oculomotor neural integrator circuit requires interactions with oculomotor neurons of the inferior olive nuclei. The presence of olivary dysplasia in three of the five autistic subjects and ectopic neurons related to the olivary complex in two cases (Bailey et al 1998) suggest that oculomotor circuitry is prone to developmental defects.

This study of the inferior olive and the cerebellar flocculus in 12 autistic and 10 control subjects revealed dysplastic changes in eight autistic (67%) and two (20%) control subjects. Focal disorganization of the cytoarchitecture, deficit and altered morphology and spatial orientation of Purkinje, granule, basket, stellate and unipolar brush cells are indicators of profound disruption of flocculus circuitry. In the flocculus of the autistic subjects, the volume of Purkinje cells was 30% less in the dysplastic than in the not-affected area ($p < 0.01$). Moreover, in the entire cerebellum of the autistic subjects the volume of Purkinje cells was 25% less than in the control subjects ($p < 0.001$). These data suggest that floccular dysplasia plays a pivotal role in dysfunction of the oculomotor system in autism.

5. Clinicopathological stratification of idiopathic autism and autism associated with duplications 15q11.2-q13

Wegiel J, Schanen NC, Cook EH, Brown WT, Kuchna I, Nowicki K, Wegiel J, Imaki H, Ma SY, London E, Wisniewski T. In: The Neuroscience of Autism Spectrum Disorders; Editors: Joseph Buxbaum and Patrick Hof, Elsevier Inc. 2013, 347-355

See Proj 1 Report (Dr. Thomas Wisniewski)

B. Contribution of altered neuronal growth to clinical autism phenotype

6. Brain-region–specific alterations of the trajectories of neuronal volume growth throughout the lifespan in autism.

Wegiel J, Flory M, Kuchna I, Nowicki K, Ma SY, Imaki H, Wegiel J, Cohen IL, London E, Brown WT, Wisniewski T. Resubmitted to Acta Neuropathologica

The prevalence of research focused on the cortex results in a mainly corticocentric theory of autism (Frith 2004, Geschwind and Levitt 2007). However, all three diagnostic modalities of autism engage subcortical structures including (a) the amygdala, in processing social information and involved in emotional interpretation, fear and anxiety (Amaral et al 2003, Baron-Cohen et al 2000, Winston et al 2002); (b) the thalamus, involved in language functions, attention, anxiety and obsessive thinking (Ojemann 1971,1977, Oke et al 1978); (c) the striatum, linked to repetitive motor behaviors, compulsions and rituals (Salamone 1944, Sears et al 1999); and (d) the brainstem and cerebellar deep nuclei, integrating a cerebellar role in motor functions, language and cognition, and eye motion control (Leyung et al 2000, Sato and Kawasaki 1991). Cholinergic neurons in the nucleus basalis of Meynert control the cortical mantle and play a modulatory role in anxiety, arousal and emotional and motor responses (Kilgard 2003, Murray and Fibiger 1985), whereas the substantia nigra's dopaminergic neurons modulate striatal functions including repetitive behaviors.

The aim of this study was to test the hypothesis that subcortical structures are affected by developmental alterations and contribute in parallel with cortical networks to global brain developmental defects of connectivity and resulting functional deficits in autism.
Several morphometric studies have revealed smaller than normal neurons in the neocortex of autistic subjects. To test the hypothesis that abnormal neuronal growth is a marker of an autism-associated global encephalopathy, neuronal volumes were estimated in 16 brain regions, including various subcortical structures, Ammon's horn, archicortex, cerebellum, and brainstem in 14 brains from individuals with autism 4 to 60 years of age and 14 age-matched control brains. This stereological study showed a significantly smaller volume of neuronal soma in all 16 regions in the 4- to 8-year-old autistic brains than in the controls. A very severe neuronal volume

deficit seen in 12.5% of examined structures, severe in 44%, moderate in 31%, and mild in 12.5% structures, suggests desynchronization of neuronal growth in the interacting neuronal networks involved in the autistic phenotype. The comparative study of the autistic and control subject brains revealed that the number of structures with a significant volume deficit decreased from 16 in the 4- to 8-year-old autistic subjects, to 6 in the 11- to 23-year-olds-, and to 5 in the 36- to 60-year-old. Neuronal volumes in 69% of the structures examined in the older adults with autism are comparable to or significantly exceeded the size of neurons in age-matched controls. This pattern suggests defects of neuronal growth in early childhood and delayed up-regulation of neuronal growth during adolescence and adulthood reducing neuron soma volume deficit. Delayed neuron growth may contribute to age-associated clinical improvements reported in some individuals with autism. However, significant correction of neuron size but limited clinical improvements suggests that delayed correction does not restore functional deficits.

7. No evidence of significant changes in the number of neurons in autistic individuals 37 brain subdivisions. Wegiel J, Flory M, Nowicki K, Kuchna I, Ma SM, Imaki H, Wegiel J, Cohen IL, London E, Brown WT, Wisniewski T. In preparation for submission.
Our prior postmortem studies of the brain of 14 autistic individuals 4 to 60-years-of age and 14 age-matched controls showed that (a) in 92% of autistic subjects at least one of three types of focal developmental defects is observed including abnormal neuronal migration, defects of neuronal proliferation and dysplasia, and (b) in all 16 examined brain regions the mean volume of neuronal soma is significantly less in 4- to 8-year-old autistic children than in control group but the size of neurons in majority of examined structures increases in adolescence and in adults to or above control level. The aim of this study of the same cohorts was to establish whether striking modifications of neuronal growth trajectories are associated with alterations of the volume of affected structures, the numerical density and the total number of neurons in these structures. In contrast to the surprisingly uniform pattern of altered neuronal growth, Cavalieri method revealed smaller volume of only one structure (n. accumbens) among 15 structures and their 21 subdivisions. Moreover, the estimates of the numerical density of neurons in 14-brain regions and their 16-cytoarchitectonic subdivisions revealed significantly lower density only in the amygdala lateral nucleus, putamen and n. accumbens of autistic subjects, whereas the fractionator revealed a lower total number of neurons only in islands in the entorhinal cortex. This stereological study of historically the largest cohort of autistic subjects and the broadest spectrum of structures, suggests a limited contribution of developmental alterations of the volume of brain structures and number of neurons to the autistic phenotype, and supports a key role of abnormal trajectory of neuron growth to behavioral changes in autism. The study of the global pattern of developmental alterations demonstrates that treatment concentrated on distortions of neuron growth may prevent/correct clinical symptoms of autism.

8. Delayed development of claustrum in autism
Jerzy Wegiel J, Janusz Morys J, Shuang Yong Ma SY, Izabela Kuchna I, Nowicki K, Imaki H, Wegiel J, Flory M, Brown WT, Wisniewski T. Chapter in preparation for submission: Functional Neuroanatomy of the Claustrum. Edited by: John Smythies, Lawrence Edelstein, V.S. Ramachandran, Elsevier 2013 (In press).

The claustrum receives inputs from many cortical areas, integrates multiple inputs into a new signal and redirects sensory information throughout the striatum and thalamus. Interconnectivity with subcortical nuclei and sensory cortical areas indicates the claustrum's

involvement in sensorimotor integration and potentially the most complex human brain function —consciousness, as well as in higher orders of functionality enabling the organism to rapidly adapt to the subtleties and nuances of a changing environment (Edelstein and Denaro 2004). The attraction to routines and sameness appears to be one of the very striking behavioral alterations characteristic of autism. It appears that claustrum immaturity, reflected in the neuronal soma deficit of 29 % in children and of 17 % in adults, and the very striking deficit of neuronal nucleus volume (42 % and 22 %, respectively), may be responsible for the claustrum neurons' functional impairment and deficits of adaptability and consciousness. The mean volume of the neuronal body in 4 to 8-year old autistic children was less by 29% (1,410 μm^3, n = 4) than in control group (1,999 μm^3; n = 4; $p < 0.000$). However, the mean volume of neuronal body in 13 to 36-year-old autistic subjects was less only by 17% (1,582 μm^3; n = 5) than in control group (1,904 μm^3; n = 5; $p < 0.000$). The mean volume of neuron nucleus in 4 to 8-year old autistic children (234 μm^3; n = 4), was 42% less than in the control group (400 μm^3; n = 4; $p < 0.000$). The mean volume of the neuronal nucleus in 13 to 36-year-old autistic subjects was 22% less (266 μm^3) than in the control group (342 μm^3). Very severe volume deficit in 4 to 8-year old autistic subjects and reduction of this deficit by 38% in cell soma volume and by 48% in the volume of nucleus volume in subjects more than 8 year old suggests delay of neuron growth in younger group and abnormal acceleration in late childhood. These data suggest also that inhibition of neuron growth is the result of altered regulation of neuron growth before age of 4 years and results in lifelong structural and functional abnormalities. Altered trajectory of neuron growth in the claustrum in comparison to other brain structures reflects both local and global dysregulation of claustrum and claustrum connectivity.

9. Different trajectories of abnormal neuronal growth desynchronize brain development in autism associated with dup15 and autism of unknown origin
Jerzy Wegiel J, Flory M, Kuchna I, Nowicki K, Ma SY, Imaki H, Wegiel J, Cohen IL, London E, Brown WT, Wisniewski T. Morphometric study in progress.

The comparison of neuron growth in dup(15) autism and in idiopathic autism reveals such common features as: 1. Developmental neuronal volume deficit, 2. Brain region specific deficits that reflect desynchronized brain development. 3. Multiregional alterations that indicate global developmental encephalopathy.

Differences between idiopathic autism and dup15 associated autism:
1. Different region specific neuron volume deficit.
2. Different trajectory of neuron growth with severe developmental delay in early childhood and significant correction in late childhood in idiopathic autism, and smaller but permanent neuron growth arrest in autism associated with dup(15).

Report will be submitted for publication before the end of 2013.

C. Contribution of metabolic alterations in neurons to clinical phenotype of autism

10. Amyloid Abnormal Intracellular Accumulation and Extracellular Aβ Deposition in Idiopathic and Dup15q11.2-q13 Autism Spectrum Disorders

Wegiel J, Frackowiak J, Mazur Kolecka B, Schanen NC, Cook EH, Sigman M, Brown WT, Kuchna I, Wegiel J, Nowicki K, Imaki H, Ma SY, Chauhan A, Chauhan V, Miller DL, Mehta PD, Cohen IL, London E, Reisberg B, de Leon MJ, Wisniewski T. PloS ONE 2012, 7, e35414.

Recent studies indicate that non-amyloidogenic cleavage of the amyloid-β peptide precursor (APP) with α and γ secretases is linked to several developmental disorders, including autism and fragile X syndrome (FXS) (Sokol et al 2006, Westmark and Malter 2007, Westmark et al 2011, Bailey et al 2008, Sokol et al 2011). The proteolytic cleavage of APP by membrane associated secretases releases several Aβ peptides possessing heterogeneous amino- and carboxyl-terminal residues including: $Aβ_{1-40}$ and $Aβ_{1-42}$ as products of β- and γ-secretases (amyloidogenic pathway); $Aβ_{17-40/42}$, as a product of α- and γ-secretases (p3 peptide, non-amyloidogenic pathway) (Iversen et al 1995, Selkoe et al 2001); and $Aβ_{pE3}$ as a product of N-terminal truncation of full length Aβ peptide by aminopeptidase A and pyroglutamate modification (Sevallae et al 2009). Aβ peptides differ in toxicity, oligomerization, fibrillization, distribution and trafficking within cells, and their contribution to Aβ deposits in plaques and vascular walls. Alzheimer disease (AD) is associated with oligomeric Aβ accumulation, fibrillar Aβ deposition in plaques, neuronal degeneration, and cognitive decline. Intraneuronal Aβ accumulation has been shown to be an early event in AD brains, and in transgenic mouse models of AD, that is linked to synaptic pathology (Gouras et al 2010, Bayer and Wirth 2010).

The aims of this comparative study of the brains of subjects with idiopathic autism (autism of unknown etiology) and with a known cause of autism [maternal dup(15)] was to test the hypothesis that regardless of the causative mechanism, autism is associated with an enhanced accumulation of Aβ in neuronal cytoplasm; (b) to show that intraneuronal Aβ is the product of non-amyloidogenic α-secretase APP cleavage ($Aβ_{17-40/42}$); (c) to show brain region and cell type-specific Aβ immunoreactivity; and (d) to identify cytoplasmic organelles involved in Aβ accumulation in the neurons of autistic and control subjects.

Enhanced $Aβ_{17-40/42}$ immunoreactivity is observed in neurons in more than 50% of subjects diagnosed with idiopathic autism. Remarkably, there is a more pronounced Aβ load in the majority of individuals diagnosed with duplications 15q11.2-q13 (dup15) and autism spectrum disorder (ASD), than in idiopathic ASD. This finding suggests early enhancement of APP processing with α-secretase. Aβ accumulation in neuronal endosomes, cathepsin D- and Lamp1-positive lysosomes and lipofuscin, as revealed by confocal microscopy, indicates that enhanced α-secretase processing is paralleled by enhanced proteolytic activity. The presence of $Aβ_{1-40/42}$ in diffuse plaques in three subjects wit ASD, 39 to 52 years of age, suggests that there is an age-associated risk of metabolic developmental alterations with an intraneuronal accumulation of a short form of Aβ and an extracellular deposition of full length of Aβ in nonfibrillar plaques. The accumulation of $Aβ_{17-40/42}$ in the astrocytes of some autistic children and adults, and in the plaque perimeter in all three plaque-positive subjects may indicate that the astrocytic cytoplasmic Aβ reflects attempted clearance and partial degradation of full length Aβ by astrocytes. The higher prevalence of Aβ alterations, early onset of intractable seizures, and a high risk of sudden unexpected death in epilepsy (SUDEP) in autistic subjects with dup(15) compared to subjects with idiopathic ASD support the concept of mechanistic and functional links between autism, epilepsy, and alterations of APP processing leading to neuronal and glial Aβ accumulation, and diffuse plaque formation.

11. The link between intraneuronal N-truncated amyloid-b peptide and oxidatively modified lipids in idiopathic autism and dup(15q11.2-q13)/autism. Acta Neuropathol, submitted

Frackowiak J, Mazur-Kolecka B, Kuchna I, Brown WT, Wegiel J.

In children with severe autism and aggression plasma levels of secreted beta amyloid precursor protein (APP) are two times higher than in children without autism and up to 4 times higher than in children with mild autism (Sokol et al 2006, Ray et al 2011). Increased levels of secreted amyloid precursor protein alpha (sAPP-α) were increased in 60% of autistic children, as compared to age-matched controls (Bailey et al 2008). Ray et al (2011) hypothesized that increased processing of APP by alpha-secretases contributes to autism. Immunocytochemical studies of the brain of autistic subjects revealed enhanced accumulation of amino-terminally truncated Aβ in cortex, subcortical structures and cerebellum (Wegiel et al 2012). Pathological effects of N-terminally truncated Aβ are not known but contribution of oxidative stress to Aβ accumulation has been suggested. In autistic subjects accumulation of intracellular Aβ in neurons correlates with colocalization with lipid peroxidation products, 4-hydroxy-2-nonenal (HNE) and malodialdehyde (MDA). It suggests that intracellular Aβ is the source of oxidative stress rather than product of oxidative stress. Therefore enhanced APP processing and intracellular Aβ accumulation might be the trigger of enhanced oxidative stress and accumulation of lipids peroxidation products with functional consequences.

KEY RESEARCH ACCOMPLISHMENTS

Project 2 integrated three major research strategies including the study of the contribution of:

1. Qualitative focal developmental abnormalities, defects of neuronal proliferation, migration and dysplastic changes in the gray matter, to the autistic phenotype.
2. Quantitative global developmental abnormalities of the trajectory of neuron growth to the autistic phenotype.
3. Developmental neuronal metabolic alterations to the clinical phenotype of autism

Major accomplishments:

1. Thanks to the DOD grant and Autism Speaks and Autism Tissue Program support for tissue acquisition, we were able to preserve historically the largest collection of unique quality brain tissue samples (72 brain hemispheres).
1. Brain hemispheres cut into serial sections provided material for several research strategies. Therefore, we were able to expand research targets and hypotheses tested in this Program Project.
2. Neuropathological component (Project #1) provided neuropathological reports and exclusion criteria reducing risk of distortion of research results by comorbidity, pre-, peri- and postmortem changes.
3. The study determined the contribution of qualitative developmental abnormalities to the autistic phenotype in autism with an unknown etiology and autism caused by maternal origin dup(15).

4. We identified both (a) differences between the pattern of developmental abnormalities in autism associated with duplications 15q11.2-q13 and autism of unknown origin and (b) core neuropathology present in autism regardless of autism etiology.
5. The project results in detection of focal abnormalities that play a key role in early onset of epilepsy, functional regression and an increased risk of Sudden Unexpected Death in Epilepsy (SUDEP).
6. This study integrates (i) localized models of defective development of neurons with (ii) more complex models of altered neuronal circuits resulting in (iii) a global model of brain development alterations.
7. Unbiased stereology of 16 brain structures and 19 anatomical subdivisions identified desynchronization of development of neurons, neuronal circuits and neurotransmitter systems as the major contributor to the autistic phenotype.
8. Mapping of these abnormalities to structures with their known role in social behavior, communication, and stereotypic behavior results in identification of a structural component of functional deficits observed in clinical studies.
9. The study suggests that the main developmental structural defect in majority of individuals with idiopathic autism are alterations of the trajectory of neuronal growth but not number of neurons, and that alterations of genes controlling neuron growth may have a major contribution to mechanisms leading to autistic phenotype.
10. Enhanced $A\beta_{17\text{-}40/42}$ immunoreactivity observed in neurons in more than 50% of subjects diagnosed with idiopathic autism, and a more pronounced Aβ load in the majority of individuals diagnosed with dup15 and autism, including children, suggests an early and significant alteration of APP processing with α-secretase.
11. The presence of $A\beta_{1\text{-}40/42}$ in diffuse plaques in three autistic subjects, 39 to 52 years of age, suggests there is an age-associated risk of metabolic developmental alterations with an intraneuronal accumulation of a short form of Aβ and an extracellular deposition of full length Aβ in nonfibrillar plaques.
12. Enhanced accumulation of intracellular Aβ in neurons correlates with colocalization with lipid peroxidation products, 4-hydroxy-2-nonenal (HNE) and malodialdehyde (MDA)and suggests that intracellular Aβ is the source of oxidative stress rather than a product of oxidative stress. Therefore, enhanced APP processing and intracellular Aβ accumulation might be a trigger of enhanced oxidative stress and accumulation of lipid peroxidation products with functional consequences.

REPORTABLE OUTCOMES

Publications

1. Wegiel J, Wisniewski T, Chauhan A, Chauhan V, Kuchna I, Nowicki K, Imaki H, Wegiel J, Ma SY, Wierzba-Bobrowicz T, Cohen IL, London E, Brown WT. Type, topography and sequelae of neuropathological changes shaping clinical phenotype of autism. In: Autism: Oxidative Stress, Inflammation, and Immune Abnormalities. Ed.: Abha Chauhan, Ved Chauhan and W. Ted Brown. Taylor & Francis/CRC Press, Boca Raton, FL, 2010, pp. 1-34.

2. Wegiel J, Kuchna I, Nowicki K, Imaki H, Wegiel J, Marchi E, Ma SY, Chauhan A, Chauhan V, Wierzba Bobrowicz T, de Leon M, Saint Louis LA, Cohen IL, London E, Brown

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3. Wegiel J, Schanen CN, Cook EH, Sigman M, Brown WT, Kuchna I, Nowicki K, Wegiel J, Imaki H, Ma SY, Marchi E, Wierzba-Bobrowicz T, Chauhan A, Chauhan V, Cohen IL, London E, Flory M, Lach B, Wisniewski T. Differences Between the Pattern of Developmental Abnormalities in Autism Associated with Duplications 15q11.2-q13 and Idiopathic Autism. J Neuropathol Exp Neurol 2012, 71, 382-397.

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7. Wegiel J, Schanen NC, Cook EH, Brown WT, Kuchna I, Nowicki K, Wegiel J, Imaki H, Ma SY, London E, Wisniewski T. Clinicopathological Stratification of Idiopathic Autism and Autism Associated with Duplications 15q11.2-q13. The Neuroscience of Autism Spectrum Disorders; Editors: Joseph Buxbaum and Patrick Hof, Elsevier Inc. Amsterdam 2013, 347-355.

8. Wegiel J, Morys J, Ma SY, Kuchna I, Nowicki K, Imaki H, Wegiel J, Flory M, Brown WT, Wisniewski T. Delayed development of claustrum in autism. Editors: John Smythies, Lawrence Edelstein, V.S. Ramachandran; Functional Neuroanatomy of the Claustrum. Elsevier In press, 2013

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10. Frackowiak J, Mazur-Kolecka B, Kuchna I, Brown WT, Wegiel J. The link between intraneuronal N-truncated amyloid-β peptide and oxidatively modified lipids in indiopathic autism and dup(15q11.2-q13)/autism. Acta Neuropathol, submitted 2013.

11. Wegiel J, Flory M, Kuchna I, Nowicki K, Ma SY, Imaki H, Wegiel J, Cohen IL, London E, Brown WT, Wisniewski T. Brain-region-specific alterations of the trajectories of neuronal volume growth throughout the lifespan in autism. Acta Neuropathol (resubmitted 2013)

12. Wegiel J, Flory M, Nowicki K, Kuchna I, Ma SM, Imaki H, Wegiel J, Cohen IL, London E, Brown WT, Wisniewski T. No evidence of significant changes in the number of neurons in autistic individuals 37 brain subdivisions. In preparation for publication.

13. Wegiel J, Flory M, Nowicki K, Kuchna I, Ma SM, Imaki H, Wegiel J, Cohen IL, London E, Brown WT, Wisniewski T. Different trajectories of abnormal neuronal growth desynchronize brain development in idiopathic autism and autism associated with dup15. In preparation for publication.

Meetings

2009: Annual International Meeting for Autism Research, Chicago, IL; May 7-9

1. Emerging patterns of neuronal growth desynchronization in autism. Wegiel J, Kuchna I, Nowicki K, Wegiel J, Ma SY, Wisniewski T, Cohen IL, London E, Flory M, Brown WT.

2. Potential contributions of developmental and epilepsy-associated neuropathological changes to sudden, unexpected death in four people with chromosome 15 duplication and autism. Brown WT, Wisniewski T, Cohen I, London E, Flory M, Kuchna I, Nowicki K, Wegiel J, Ma SY, Imaki H, Wegiel J.

3. Delayed development of neurons in networks involved with stereotypic behaviors and reward in autism. Nowicki K, Kuchna I, Wegiel J, Ma SY, Wisniewski T, Cohen IL, London E, Flory M, Brown WT, Wegiel J.

4. Developmental heterochronicity of neuron growth in the memory system of autistic subjects. Kuchna I, Nowicki K, Wegiel J, Ma SY, Wisniewski T, Cohen IL, London E, Flory M, Brown WT, Wegiel J.

5. Contribution of thalamic developmental changes to the autistic phenotype. Ma SY, Kuchna I, Nowicki K, Wegiel J, Wisniewski T, Cohen IL, London E, Flory M, Brown WT, Wegiel J.

2010: Annual International Meeting for Autism Research, Philadelphia, PA; May 20-22

6. Defects of neurogenesis, neuronal migration and dysplastic changes in the brains of autistic subjects. Wegiel J, Kuchna J, Nowicki K, Imaki H, Wegiel J, Marchi E, Ma SY, Chauhan A, Chauhan V, Cohen IL, London E, Brown WT, Wisniewski T.

7. Repetitive and stereotyped behaviors in autism are driven by abnormal development of the striatum but not of the substantia nigra. Nowicki K, Wisniewski T, Kuchna I, Wegiel J, Imaki H, Ma SY, Cohen IL, London E, Flory M, Brown WT, Wegiel J.

8. Neuronal growth delay within the claustrum of autistic subjects. Ma SY, Kuchna I, Nowicki K, Wegiel J, Imaki H, Cohen IL, London E, Flory M, Brown WT, Wisniewski T, Wegiel J.

2011: Annual International Meeting for Autism Research, San Diego, CA, May 12-14

9. Neuropathology of idiopathic autism and autism associated with chromosome 15 duplication. Wegiel J, Kuchna I, Nowicki K, Ma SY, Wegiel J, Frackowiak J, Mazur Kolecka B, Marchi E, Cohen IL, London E, Brown WT, Wisniewski T.

10. The olivo-floccular circuitry developmental defects in autism. Kuchna I, Imaki H, Nowicki K, Ma SY, Wegiel J, Cohen IL, London E, Flory M, Brown WT, Wisniewski T, Wegiel
11. Hypothalamic neurons developmental delay in autistic subjects. Ma SY, Kuchna I, Nowicki K, Wegiel J, Imaki H, Cohen IL, London E, Flory M, Brown WT, Wisniewski T, Wegiel J.

12. Defects of cholinergic neurons development in autism. Nowicki K, Kuchna I, Ma SY, Wegiel J, Imaki H, Cohen IL, London E, Flory M, Brown WT, Wisniewski T, Wegiel J.

13. Accumulation of amyloid-beta peptide species in four brain structures in children with autism. Frackowial J, Mazur Kolecka B, Izabela K, Nowicki K, Brown WT, Wisniewski T, Wegiel J.

2012: NIH meeting, Dup14/autism Alliance meeting, Cell Development Conference

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15. Clinicopathological stratification of idiopathic autism and autism associated with duplications 15q11.2-q13. Wegiel J, Schanen NC, Cook EH, Brown WT, Kuchna I, Nowicki K, Wegiel J, Imaki H, Ma SY, London E, Wisniewski T. Dup15q Alliance 2012 Scientific Meeting. Boston Children's Hospital, Boston August 9-10, 2012

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2013: Annual International Meeting for Autism Research, San Sebastian, Spain, May 3-5

17. Global pattern of delayed and desynchronized neuron growth in the brain of autistic subjects. J. Wegiel, M. Flory, I. Kuchna, K. Nowicki, S.Y. Ma, H. Imaki, J. Wegiel, I.L. Cohen, E. London, T. Wisniewski, W.T. Brown

18. Abnormalities in Raphe Nuclei of Autistic 5 to 15 Year Old Subjects. Jarek Wegiel, Efrain C. Azmitia, Thomas Wisniewski, Probal Banerjee

CONCLUSIONS

A. Developmental alterations as a contributor to enhanced prevalence of epilepsy in idiopathic autism. One of the first studies revealed that in 92% of cases with autism at least one of three types of focal developmental abnormalities is observed, including defects of neuronal migrations resulting in heterotopias, defects of neuronal proliferation with subependymal nodular dysplasia, and dysplastic changes in the cortex and subcortical structures. The type, topography and severity of alterations suggest that they contribute to enhanced prevalence to seizures and SUDEP in idiopathic autism [Wegiel et al 2010].

B. Increased prevalence of developmental defects, epilepsy and SUDEP in dup15/autism. This concept was confirmed and expanded in the study of focal dysplasia and heterotopias in dup 15/autism documenting that these abnormalities are a major cause of the increased risk of sudden unexpected death in early childhood in autism associated with dup15

1. Severe microcephaly, with brain weight reduced by 300 g is the major sign of global encephalopathy increasing the risk of epilepsy in dup15/autism
2. 2.8 times more frequent developmental alterations, especially common in the hippocampal formation of autistic subjects with dup15, and presence of up to 11 different types of developmental alterations are the major contributor to early onset of epilepsy and high risk of SUDEP.
3. Combination of all of these developmental defects increases risk of death at a very early age (~10 years) in autism associated with dup15.

C. Delayed, desynchronized growth of neurons in all examined cortical and subcortical structures is the major marker of developmental defects contributing to the autism phenotype

1. **Marker of global brain encephalopathy**. To test the hypothesis that abnormal neuronal growth is a marker of an autism-associated global encephalopathy, neuronal volumes were estimated in 16 brain regions, including various subcortical structures, Ammon's horn, archicortex, cerebellum, and brainstem. This study revealed that idiopathic autism is associated with a significantly smaller volume of neuronal soma in all 16 examined regions in the 4- to 8-year-old autistic children than in controls suggesting global developmental encephalopathy.
2. **Desynchronized development of neurons in interacting networks**. A very severe neuronal volume deficit in 12.5% of examined structures, severe in 44%, moderate in 31%, and mild in 12.5% structures is a sign of desynchronized development of anatomically and functionally related neurons. These abnormalities may contribute to social and communication deficits, and restricted repetitive and stereotyped patterns of behavior.
3. **Delayed up-regulation of neuron growth**. Altered trajectories of neuron growth with a decrease in the number of brain structures with significantly smaller neurons from 16 in the 4- to 8-year-old autistic subjects, to 6 in the 11- to 23-year-olds-, and to 5 in the 36- to

60-year-olds reflect delayed up-regulation of neuronal growth during adolescence and adulthood reducing neuron soma volume deficit.

4. **Significant increase of neuron size but limited clinical improvement**. It suggests that delayed correction does not fully normalize neuron structure and functions.

D. No evidence of altered number of neurons in 37 brain subdivisions in autistic subjects.

1. In contrast to published reports showing a local increase, decrease or no changes in the number or numerical density of neurons, the application of the fractionator revealed that in all 14-brain regions and 23- subdivisions (cortical layers, hippocampal sectors, and nuclei) examined, the numerical density and total number of neurons per region of interest does not differ significantly in autistic and control subjects.

2. This strikingly strong pattern of changes of neuronal growth trajectory but no evidence of changes of the number of neurons was detected in the largest group of autistic individuals examined postmortem and in the broadest spectrum of structures examined with unbiased stereological methods.

3. The list of structures with unmodified number of neurons includes the amygdala, thalamus, four striatal subdivisions, substantia nigra, magnocellular basal complex, lateral geniculate nucleus, Ammons horn, entorhinal cortex, dentate nucleus and inferior olive.

4. The study suggests that the main developmental structural defects in the majority of individuals with idiopathic autism are alterations of the trajectory of neuronal growth but not the number of neurons, and that alterations of genes controlling neuron growth may have a major contribution to mechanisms leading to the autistic phenotype.

E. Metabolic alterations reflected in enhanced accumulation of truncated Aβ and oxidative stress contribute to the autistic phenotype

1. Abnormal accumulation of amino-terminally truncated Aβ in neurons and glial cells is a common finding in the brain of autistic children and young adults.
2. Enhanced accumulation is brain region and cell type-specific.
3. These developmental alterations are more severe in brains of idic15 subjects diagnosed with autism and epilepsy than in idiopathic autism.
4. The presence of Aβ in lysosomes, autophagic vacuoles and lipofuscin, as well as presence of Aβ not associated with these structures, suggests that enhanced intracellular accumulation of Aβ in autism results from different pathways of APP processing and Aβ deposition.
5. Detection of increased levels of Aβ complexes in the soluble and insoluble form in the brain cortex and cerebellum of autistic subjects indicates brain structure-specific alteration of APP processing and Aβ trafficking in autism.
6. Abnormalities of Aβ intracellular accumulation in early stage of brain development suggest their link to the clinical phenotype, including seizures, in idiopathic autism and autism associated with idic15.
7. Aβ accumulation in neurons initiates oxidative stress resulting in lipid peroxidation. Accumulation of Aβ in activated astrocytes results in their death. Both are markers of

developmental alterations in APP processing with structural and probably functional consequences.

F. The link between intraneuronal N-truncated amyloid-β peptide and oxidatively modified lipids in indiopathic autism and dup(15q11.2-q13)/autism

1. Our previous study revealed enhanced accumulation of amino-terminally truncated amyloid-β (Aβ) in brain neurons and glia in children and adults with autism. To verify the hypothesis that intraneuronal Aβ causes oxidative stress, the relationships between neuronal Aβ and oxidative stress markers —4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA)—were examined in the frontal cortex from individuals aged 8–32 years with idiopathic autism or with chromosome 15q11.2-q13 duplications (dup(15)) with autism, and age-matched controls.
2. Quantification of confocal microscopy images revealed significantly higher levels of neuronal N-truncated Aβ and HNE and MDA in idiopathic autism and dup(15)/autism than in controls. Lipid peroxidation products were detected in all mitochondria and lipofuscin deposits, in numerous autophagic vacuoles and lysosomes, and in less than 5% of synapses.
3. Neuronal Aβ was co-localized with HNE and MDA, and increased Aβ levels correlated with higher levels of HNE and MDA. The results suggest a self-enhancing pathological process in autism that is initiated by intraneuronal deposition of N-truncated Aβ in childhood.
4. The cascade of events includes altered APP metabolism and abnormal intracellular accumulation of N-terminally truncated Aβ which is a source of reactive oxygen species, which in turn increase the formation of lipid peroxidation products.
5. This process enhances Aβ deposition and sustains the cascade of changes contributing to metabolic and functional impairments of neurons in autism of an unknown etiology and caused by chromosome 15q11.2-q13 duplication.

G. The link between developmental alterations in the oculomotor system and abnormal gaze in autism

1. The flocculus and the inferior olive are the components of the olivo-cerebellar system involved in control of oculomotor function and gaze control. The study revealed that the flocculus is affected by dysplastic changes in 67% of autistic subjects.
2. Disorganization of the granule, molecular and Purkinje cell layer, striking deficit of Purkinje cells, their abnormal spatial orientation, severe deficit and distortion of the Purkinje cells' dendritic tree are the major structural defects in the dysplastic portion of the flocculus.
3. The volume of Purkinje cells in the flocculus of control subjects is 20% less (8,865 um^3) than in other parts of the cerebellar cortex (11,092 um^3, $p<0.03$). In autistic subjects the volume of Purkinje cells is significantly less than in control subjects in the entire cerebellar cortical ribbon ($p<0.001$).
4. Severe developmental abnormalities in the flocculus combined with reduced volume of Purkinje cells in the entire cerebellum of autistic subjects but no changes in morphology and neuronal size in the inferior olive, the second component of the olivo-floccular integrator of oculomotor function, suggests that mechanism leading to floccular dysplasia may play a pivotal role in defective function of the oculomotor system in autism.

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APPENDICES

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1 Type, Topography, and Sequelae of Neuropathological Changes Shaping Clinical Phenotype of Autism

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1.1 INTRODUCTION

The aim of this chapter is to identify the type, topography, and sequelae of neuropathological changes that contribute to the clinical phenotype of autism. Results of recent magnetic resonance imaging (MRI) and postmortem neuropathological and stereological studies of autism brain suggest a dynamic model of sequential subdivision of age- and brain-specific structural and functional changes. Acceleration of brain growth in the first year of life and deceleration in the second and third years appear to play a pivotal role in the onset of clinical signs of autism (Courchesne and Pierce, 2005b; Courchesne et al., 2001, 2003; Dawson et al., 2007; Dementieva et al., 2005; Gillberg and de Souza, 2002; Redcay and Courchesne, 2005). The range of deviation from the normal trajectory of brain growth may be a factor determining the severity of the disease (Courchesne et al., 2003). Developmental heterochronicity (differential rates of growth of various brain regions compared to controls), resulting in selective overgrowth of some brain

regions, appears to be a key factor determining topography and brain region–specific type of cytoarchitectonic changes (Carper and Courchesne, 2005; Carper et al., 2002; Courchesne et al., 2001; Hazlett et al., 2005; Sparks et al., 2002). Topographic developmental heterochronicity may result in impairment of both local and global connectivity, leading to local overconnectivity and impairment of long-distance connectivity (Baron-Cohen, 2004; Casanova et al., 2006; Courchesne and Pierce, 2005a). Stereological studies have revealed neuronal developmental heterochronicity in early childhood, resulting in selective developmental delay of the growth of neurons in some subcortical structures and the cerebellum during the most critical stage of development of social behaviors and communication skills (Wegiel et al., 2008). Distortions of brain and neuronal development are reflected in abnormal cortical minicolumn organization (Casanova et al., 2002, 2006), local dysgenesis, and ectopias (Bauman and Kemper, 1985; Bauman et al., 1997; Kemper and Bauman, 1993, 1998). These complex developmental abnormalities appear to lay the foundation for secondary and tertiary metabolic, structural, and functional changes, including seizures and risk of sudden unexpected death; signs of oxidative stress, early and enhanced accumulation of products of cell organelle degradation with lipofuscin deposition; modified processing of β-amyloid precursor protein with accumulation of truncated amyloid beta; and other as of yet unidentified changes. Secondary pathologic changes appear to be indicators of the susceptibility of abnormally developing neurons to further modifications during cell maturation and aging. The pattern of morphological changes emerging from these multidisciplinary studies appears to represent a major trend. However, modifications of the course of disease and subpatterns of developmental changes result in a broad spectrum of morphological and clinical interindividual differences.

1.2 CLINICAL, ETIOLOGICAL, AND NEUROPATHOLOGICAL DIVERSITY IN AUTISM

Autism is the prototype of a pervasive developmental disorder (PDD) and is characterized by (a) qualitative impairments in reciprocal social interactions, (b) qualitative impairments in verbal and nonverbal communication, (c) restricted repetitive and stereotyped patterns of behavior, interests, and activities, and (d) onset prior to the age of 3 years. PDD also includes childhood disintegrative disorder, Asperger's disorder, Rett syndrome, and pervasive developmental disorder—not otherwise specified (PDD-NOS). The common features of all these disorders are qualitative deficits in social behavior and communication (American Psychiatric Association, 2000).

1.2.1 Clinic

In most cases (90%–95%), it is not presently possible to detect a known or specific etiology. These cases are referred to as idiopathic or nonsyndromic autism (Boddaert et al., 2009; Gillberg and Coleman, 1996). In 6% (Fombonne, 2003), 5% (Tuchman et al., 1991), or 10% (Rutter et al., 1994) of cases, autism was diagnosed in association with other disorders. About 30% of children with idiopathic autism have complex autism, defined by the presence of dysmorphic features, microcephaly and/or a structural brain

malformation (Miles et al., 2005). About 70% of children with autism have essential autism, defined by the absence of physical abnormalities. For most children, the onset of autism is gradual. However, a multisite study revealed significant regression at ages of 18 to 33 months (regressive autism) in about 13.8% (Colorado) to 31.6% (Utah) of autistic subjects (Department of Health and Human Services, 2007). Moreover, the manifestations of autism vary greatly, depending on developmental level and chronological age of the affected individual. The majority of patients exhibit serious social and communicative impairments throughout life but some improve enough to be able to live relatively independently as adults. In 44.6% of children, autism is associated with cognitive impairment (defined as having intelligence quotient scores of <70; Department of Health and Human Services, 2007). Expressive language function in individuals with autism may vary from mutism to verbal fluency (Rapin, 1996; Stone et al., 1997; Wetherby et al., 1998). Sensorimotor deficits also show significant interindividual differences, with more frequent and severe impairments of gross and fine motor function (motor stereotypes, hypotonia, limbic apraxia) in subjects with lower IQ (Rogers et al., 1996). Hand mannerisms and body rocking are reported in 37% to 95% of individuals with autism (Lord and Rutter, 1995; Rapin, 1996; Rogers et al., 1996), whereas preoccupation with sensory features of objects, abnormal responsiveness to environmental stimuli, or paradoxical responses to sensory stimuli are seen in 42% to 88% of people with autism (Kientz and Dunn, 1997). Epilepsy is a comorbid complication, occurring in up to 33% of individuals with autism (Tuchman and Rapin, 2002).

1.2.2 Etiology

The clinical diversity of autism reflects the etiologic heterogeneity of this disorder. Genetic factors; pre-, peri-, and postnatal pathological factors; and concurrent diseases may contribute to autism (Muhle et al., 2004; Newschaffer et al., 2002; Rutter et al., 1994). About 5% to 10% of cases are associated with several distinct genetic conditions including fragile X syndrome, tuberous sclerosis, phenylketonuria, Rett syndrome, and chromosomal anomalies such as Down syndrome (DS) (Folstein and Rosen-Scheidley, 2001; Fombonne, 2003; Smalley et al., 1988; Yonan et al., 2003). Autism spectrum disorders (ASDs) in people with DS have been described in several reports (Ghaziuddin et al., 1992; Howlin et al., 1995; Prasher and Clarke, 1996; Wakabayashi, 1979), and the prevalence of autism in boys with DS was estimated as at least 7% (Kent et al., 1999). The prevalence of autism in the fragile X syndrome is estimated as 15%–28% (Hagerman, 2002). Cytogenetic abnormalities (partial duplications, deletions, inversions) in the 15q11-q13 region account for 1% to 4% of autism cases (Cook, 1998; Gillberg, 1998). Several potential candidate genes have been identified in both autosomes and X chromosomes, including the tuberous sclerosis gene on chromosomes 9 and 16; serotonin transporter on chromosome 17; gamma-aminobutyric acid receptor-beta 3 on chromosome 15; neuroligins on the X chromosome (see Vorstman et al., 2006); and possibly *PTEN* on chromosome 10 (Butler et al., 2005). Modifications in the tryptophan hydroxylase gene may play a modest role in autism susceptibility (Coon et al., 2005).

1.2.3 Neuropathology

While knowledge of the clinical and genetic factors in autism is based on examination of thousands of patients, postmortem neuropathological studies are based on reports of a very small number of brains. A review by Palmen et al. (2004) revealed that between 1980 and 2003, only 58 brains of individuals with autism have been examined, and results of only a few neuropathological and stereological studies were published. Usually, neuropathological reports and morphometric reports were based on evaluation of one or several brains. Due to the broad age spectrum and the etiological and clinical diversity in autism, the pattern of neuropathological changes reported is incomplete and often inconsistent. As a result, the morphological markers and neuropathological diagnostic criteria of autism have not yet been established (Lord et al., 2000; Pickett and London, 2005). In the past, the contribution of postmortem studies to the detection and characterization of neuropathological changes and mechanisms leading to structural and functional manifestations of autism was limited because of (a) the deficit of autism brains, resulting in a lack of statistical power, (b) the lack of efficient mechanisms for sharing the limited tissue resources, (c) the lack of complex studies of the dynamic of changes during the life span, (d) the infrequent application of unbiased morphometric methods to detect quantitative differences, and (e) the averaging of results from subjects with different clinical and morphological manifestations of autism. Heterogeneity within the autism spectrum is the major obstacle to autism research at all levels (Newschaffer et al., 2002), including neuropathological studies and attempts at detection of clinicopathological correlations. Recent evidence of genetic fractionation of social impairment, communication difficulties, and rigid and repetitive behaviors indicates that heterogeneity in ASD could be an unavoidable consequence of the contribution of nonoverlapping genes. If different features of autism are caused by different genes associated with different brain regions and related to different core cognitive impairments (Happe et al., 2006), it seems likely that many brain networks are involved in the pathology of autism. The diversity of neuropathological findings and the commonly reported inconsistencies in regional findings correspond to developmental impairments in many interacting brain networks and to expansion from "local" abnormalities to "nonlocal" effects of the emerging cognitive system. In spite of these limitations, "localizing" models are still the main approach to the identification of pathological changes as a component of the structural and functional abnormalities of the networks (Müller, 2007).

The possibility that autism is associated with neuropathological changes was explored in the first studies reported between 1980 and 1989 (Bauman and Kemper, 1985; Courchesne et al., 1987, 1988; Damasio et al., 1980; Gaffney et al., 1987; Hashimoto et al., 1989, 1993; Murakami et al., 1989; Ritvo et al., 1986). Expansion of these studies through examination of larger cohorts and application of stereology, functional and structural MRI, and biochemistry resulted in the identification of several major forms of pathology contributing to the clinical phenotype including abnormal acceleration of brain growth in early childhood (Redcay and Courchesne, 2005), delay of neuronal growth (Wegiel et al., 2008), changes in brain cytoarchitecture (Bailey et al., 1998; Bauman and Kemper, 1985; Casanova et al., 2002, 2006),

metabolic modifications with abnormal amyloid precursor protein (APP) processing (Bailey et al., 2008; Brown et al., 2008; Sokol et al., 2006), enhanced oxidative stress (reviewed in Chauhan and Chauhan, 2006), and turnover of cell organelle with pigment accumulation and glial activation (Lopez-Hurtado and Prieto, 2008).

1.3 DEREGULATION OF BRAIN GROWTH IN EARLY CHILDHOOD

The major measures of age-related changes are head circumference, MRI-based volumetry of the brain and brain structures, and postmortem brain weight and volume of brain subdivisions. Between 1990 and 2000, several groups reported increased head circumference (Bailey et al., 1995; Bolton et al., 1995; Davidovitch et al., 1996; Fidler et al., 2000; Fombonne et al., 1999; Lainhart et al., 1997; Miles et al., 2000; Steg and Rapoport, 1975; Stevenson et al., 1997), whereas MRI-based studies revealed increased brain volume (Piven et al., 1995, 1996). According to Fombonne et al. (1999), the prevalence of macrocephaly in autism is about 20%. In a report by Bailey et al. (1998), four of six subjects with autism 4 to 24 years of age had macrocephaly. Increased brain weight was reported in postmortem studies by Bailey et al. (1993) and Kemper and Bauman (1998). Increase in the volume was regional and not generalized, with the greatest enlargement in the occipital and parietal lobes (Filipek, 1996; Filipek et al., 1999; Piven et al., 1995, 1996). However, in several studies, an increase in brain size was not detected (Garber and Ritvo, 1992; Haznedar et al., 2000). Inconsistency in detection of abnormal head and brain size can be associated with interindividual differences, the age of examined individuals, and the methods applied. Courchesne et al. (2003) integrated their work and that of other researchers into the concept of four phases of modified brain growth, described below.

At birth, the head circumference of neonates later diagnosed with autism is normal or slightly less than that observed in normally developing children (Courchesne et al., 2003; Dawson et al., 2007; Dementieva et al., 2005; Dissanayake et al., 2006; Gillberg and de Souza, 2002; Hazlett et al., 2005; Lainhart et al., 1997; Mason-Brothers et al., 1990; Stevenson et al., 1997). Slight undergrowth is independent of body growth and may be a reflection of prenatal neural developmental defects corresponding to pathology detected in postmortem studies of the brains of autistic adults (Bailey et al., 1998; Casanova et al., 2002; Courchesne et al., 2003; Kemper and Bauman, 1998). In only 5% of neonates diagnosed later as autistic was the head circumference more than that in normally developing infants (Courchesne et al., 2003; Dementieva et al., 2005).

In the second phase, by 1 or 2 years of age, a rapid and large increase in head circumference distinguished children diagnosed later with autism from normally developing children (Courchesne et al., 2003; Dawson et al., 2007; Dementieva et al., 2005; Dissanayake et al., 2006; Hazlett et al., 2005). Ninety percent of 2- and 3-year-old children with autism had brain volumes larger than those of control children (Courchesne at al., 2001). According to Dawson et al. (2007), a period of exceptionally rapid head growth is limited to the first year of life, and head growth decelerates after 12 months of age. Acceleration of growth in head circumference appears to begin at about 4 months (Courchesne and Pierce, 2005a; Gillberg and

de Souza, 2002; Redcay and Courchesne, 2005). Using meta-analysis based on evaluation of head circumference converted to brain volume, brain volume measured from MRI, and brain weight from postmortem studies, Redcay and Courchesne (2005) revealed that brain size increases from 13% smaller than in control subjects at birth to 10% larger than in control infants at 1 year, but only 2% greater by adolescence. The greater growth rate of head circumference in the first year, and its return to normal rates thereafter, is not accounted for by an overall growth in stature. Studies of behavioral development in infants later diagnosed with autism suggest that the period of acceleration of head growth precedes and overlaps with the onset of behavioral changes, and that the period of deceleration coincides with a period of behavioral decline or worsening of symptoms in the second year of life (Dawson et al., 2007). Coincidence of acceleration of brain growth rate with onset and worsening of clinical symptoms may indicate that structural developmental changes critical for a lifelong phenotype occur in early infancy. Acceleration of brain growth in the first year and deceleration in the second year of life suggest that failure of the mechanism controlling brain growth in the first year of life plays an essential role in the onset of clinical features of autism. Identification of these mechanisms may lead to conceptualization of early preventive treatments.

In the third phase, of 2 to 4 years, the overall rate of brain growth slows but is still 10% more than in normally developing children (Carper et al., 2002; Courchesne et al., 2001; Hazlett et al., 2005; Sparks et al., 2002). In 4- to 5-year-old autistic children, MRI-based estimated brain volume is 1350 mL, whereas in normally developing children, a comparable volume (1360 mL) is reached about 8 years later. In postmortem studies, the brain weight of 3- to 5-year-old autistic males was 15% higher (1451 g) than in control males of this age (1259 g) (Redcay and Courchesne, 2005).

In the fourth phase, the volume of the brain decreases, and this trend extends from middle/late childhood through adulthood. Head (Aylward et al., 2002) or brain enlargement (Bailey et al., 1998; Hardan et al., 2001; Lainhart et al., 1997; Piven et al., 1995, 1996) has also been observed in studies of older populations of autistic individuals. However, by adolescence and adulthood, the average size of the brain is only 1% to 3% greater in autistic than in control cohorts (Redcay and Courchesne, 2005).

Moreover, the pattern of brain growth reflects the severity of clinical manifestation of autism (Courchesne et al., 2003). Among infants who have the more severe form of autism, 71% showed increases during their first year of more than 1.5 S.D., with 59% showing increases between 2.0 and 4.3 S.D. In children with a less severe form of autism, PDD-NOS, acceleration of brain growth is observed later, and the increase is less pronounced. Later onset and slower rate of progression of autism appear to be associated with a better outcome.

1.3.1 Developmental Heterochronicity

Developmental heterochronicity studies indicate that autism is a disorder involving a transient period of pathological acceleration of brain growth. Developmental heterochronicity, with different rates of growth for different brain regions/structures, appears to be the second major factor contributing to the clinical phenotype. MRI studies showed that overgrowth of the frontal and temporal lobes and amygdala, brain

regions that are involved in cognitive, social, and emotional functions as well as language development, is synchronized with brain overgrowth in 2- to 4-year-old autistic children in contrast to a different rate of growth of the occipital cortex (Carper and Courchesne, 2005; Carper et al., 2002; Courchesne et al., 2001; Hazlett et al., 2005; Sparks et al., 2002). The reduced size of the body and posterior subregions of the corpus callosum noted in subjects with autism may indicate disproportions in brain subregions development (Piven et al., 1997b). The cellular and molecular basis for transient acceleration of brain growth and enhanced growth of some brain regions is not known, but Courchesne et al. (2003) proposed that the observed pattern is associated with an excessive number of neurons, enhanced rate of growth of size of neurons, and increased number of minicolumns as well as excessive and premature expansion of the dendritic tree.

1.3.2 Functional Consequences of Abnormal Brain Development

Using a computational model analogue of autism, Cohen (2007) has argued that an interaction between stochastic and above-average or "excessive" numbers of neural connection factors has implications for understanding the disorder. In particular, a relative excess of connections could lead to enhanced recognition of complex patterns in the environment. In Cohen's chapter, it was noted that if large and complex brains are in part familial (Courchesne et al., 2003; Fidler et al., 2000), and brain size is heritable (Pfefferbaum et al., 2000) and positively correlated with IQ (Pennington et al., 2000), then behavioral outcomes both within and across generations of family members could result in (a) individuals who may be unusually gifted in their ability to handle complex nonlinear problems such as mathematics or computer science, (b) individuals with autism, or (c) individuals with a combination of autism or autistic-like behavior and giftedness (many typical Asperger's cases). These trends are detected among relatives of subjects with autism (Folstein and Rutter, 1988).

The effect of an abnormal trajectory of brain development observed in autism are well-validated characteristics of the learning style of children with autism, including (a) greater attention to idiosyncratic than socially relevant stimuli, (b) stimulus overselectivity or a lack of drive for central coherence, (c) problems with acquiring fuzzy concepts, (d) development of savant skills, (e) problems with generalization of previously acquired skills, (f) rigidity and resistance to change, (g) social and communication deficits, and (h) difficulty in learning complex higher-order concepts (Cohen, 2007).

1.4 CORTICAL AND SUBCORTICAL NEUROPATHOLOGY

1.4.1 Cortical Dysgenesis, Lamination Defects, Migration Disturbances

The fundamental characteristics of the neuropathological changes described by Kemper and Bauman (1993, 1998), Bauman and Kemper (1985, 1996), and Bauman et al. (1997) suggest three major neuropathologies in the brain of people with autism: (a) curtailed development of neurons in the structures that are substrates for memory and emotions—the entorhinal cortex, hippocampus, subiculum, anterior cingulate

gyrus and mamillary body; (b) a congenital decrease in the number of Purkinje cells in the cerebellum; and (c) age-related differences in cell size and number of neurons in the cerebellar nuclei and in the inferior olivary nucleus. Microdysgenesis is represented by increased neuronal density in the cortical layer, clustering of cortical neurons, disorganization of cortical layers, neuron cytomegaly, ectopic neurons, and nodular heterotopias. A detailed study of serial sections from the brain of a 29-year-old man with autism revealed reduced neuronal size and increased cell-packing density (Bauman and Kemper, 1985), both features of an immature brain (Friede, 1975). Cell-packing density was increased by 66% in the hypothalamus and mamillary body, and by 54% in the medial septal nucleus, with smaller nerve cells. The reduced size of neurons and the selective increase in cell-packing density were seen in central (40%), medial (28%), and cortical nuclei (35%). Atrophy of the neocerebellar cortex, with marked loss of Purkinje cells and, to a lesser extent, of granule cells, was present in gracile, tonsil, and inferior semilunar lobules. Changes were not detected in the anterior lobe or the vermis. Reduced numbers of cells were noticed in fastiglial, globose, and emboliform nuclei, and cells were small and pale. The dentate nucleus was distorted. Retrograde neuronal loss in the inferior olive related to neuronal loss in cerebellar cortex was not found, but olivary neurons were small and pale. Brain cytoarchitecture abnormalities were not associated with gliosis. In a 21-year-old female with autism, Rodier et al. (1996) found that the brain was smaller than a control brain, and the length of facial nerve nucleus was less than 500 μm as compared to 2610 μm in the control subject.

1.4.2 Brain Structure–Specific Delay of Neuronal Growth

The reduced size of neurons and their nuclei in the cortex of autistic subjects reported by Casanova et al. (2006) could be an indicator of reduced or impaired functional connectivity between distant cortical regions (Casanova et al., 2006; Just et al., 2004; Koshino et al., 2005). Our ongoing studies of series of brains from age-matched autistic and control subjects (Wegiel et al., 2008) indicate that reduced size of neurons is a brain structure–specific marker. In 4- to 7-year-old autistic children, Purkinje cells were smaller by 38%. Neurons in the dentate nucleus were reduced by 26%; in the amygdala, by 24%; in the nucleus accumbens, by 41%; in caudate, by 20%; and in the putamen, by 27%. Neurons in the nucleus of the facial nerve and the nucleus olivaris did not show a significant difference from controls. The second significant feature of the pattern of neuronal size abnormalities is the partial or complete correction of the size of neurons (for example, in the nucleus accumbens) observed in late childhood or adulthood. This study indicates that the delay of growth of neurons is the most consistent pathology detected in the brains of examined people with autism. Pathology is brain structure–specific. Changes may range from no delay to severe developmental delay. The youngest examined children (4 to 7 years old) show the most severe deficit in the volume of the neuronal body and nucleus. Partial correction of cell volume is observed in late childhood and adulthood, which indicates that brain structure and function undergo modifications during the life span. The study of basal ganglia and cerebellum supports the hypothesis that clinical manifestations of autism are the result of regional neuronal maldevelopment.

One may assume that mechanisms regulating growth of the neuron in early childhood are the target of factors that are the cause of autism. The result of deregulation of these mechanisms could be (a) significantly delayed growth of neuronal body, nucleus, dendritic tree, spines, and reduced number of synapses and (b) functional deficits corresponding to these structural developmental delays. These abnormalities of very early childhood might be the major contributor to clinical deficits that are the basis for the clinical diagnosis of autism at the age of 3 years.

1.4.3 Minicolumnar Abnormalities in Autism

The next significant contribution to detection of neocortical developmental pathology is the result of studies of minicolumns by Casanova's group (Buxhoeveden and Casanova, 2002; Casanova et al., 2002, 2006). Malformations of cortical development are observed in heterogeneous disorders caused by abnormalities of cell proliferation, apoptosis, cell migration, cortical organization, and axon pathfinding (Hevner, 2007). Clinically malformations of cortical development are significant causes of mental retardation, seizures, cerebral palsy, and neuropsychiatric disorders (Barkovich et al., 2005; Guerrini and Marini, 2006; Sarnat and Flores-Sarnat, 2004). Minicolumns are considered a basic architectonic and functional unit of the human neocortex (Buxhoeveden and Casanova, 2002; Casanova et al., 2002). Increased neuron density by 23%, reduced size of neurons in minicolumns, and a concomitant increase in the total number of minicolumns appears to illustrate the bias of local rather than global information processing (Casanova et al., 2002, 2006), resulting in a "hyper-specific brain" (McClelland, 2000). Synchronization of interactions requiring the involvement of distant brain regions is impaired in autism as a result of developmental connectivity deficits (underconnectivity) of smaller neurons (Just et al., 2004; Koshino et al., 2005; Zilbovicius et al., 1995). Structural imaging studies also suggest the overrepresentation of short association fibers in autism, with a regional increase in the volume of white matter (Herbert et al., 2004) favoring the local information processing observed in autistic subjects (Happe, 1999).

1.5 NEURONAL OXIDATIVE STRESS AND METABOLIC CHANGES

An increasing body of evidence suggests that the abnormal rate of development of neurons and neuronal networks in early infancy is followed by metabolic changes, with signs of oxidative stress, enhanced autophagocytosis, and lipofuscin accumulation, leading to early selective neuronal structural and functional changes.

1.5.1 Oxidative Stress in Autism

Oxidative stress is known to be associated with premature aging of cells and can lead to inflammation, damaged cell membranes, autoimmunity, and cell death. The brain is highly vulnerable to oxidative stress due to its limited antioxidant capacity, higher energy requirement, and high amounts of unsaturated lipids and iron (Juurlink and Peterson, 1998). The brain makes up about 2% of body mass but consumes 20% of metabolic oxygen. The vast majority of energy is used by the neurons (Shulman et al., 2004). Glutathione (GSH) is the most important antioxidant for detoxification

and elimination of environmental toxins. Due to the lack of glutathione-producing capacity by neurons, the brain has a limited capacity to detoxify reactive oxygen species (ROS). Therefore, neurons are the first cells to be affected by the increase in ROS and shortage of antioxidants and, as a result, they are most susceptible to oxidative stress. Antioxidants are required for neuronal survival during the early critical period (Perry et al., 2004). Children are more vulnerable than adults to oxidative stress because of their naturally low glutathione levels from conception through infancy (Erden-Inal et al., 2002; Ono et al., 2001). The risk created by this natural deficit in detoxification capacity in infants is increased by the fact that some environmental factors that induce oxidative stress are found at higher concentrations in developing infants than in their mothers, and accumulate in the placenta.

Accumulating evidence from our and other groups suggests increased oxidative stress in autism (reviewed in Chauhan and Chauhan, 2006). Lipid peroxidation is a chain reaction between polyunsaturated fatty acids and ROS, producing lipid peroxides and hydrocarbon polymers that are both highly toxic to the cell. We have reported that levels of malondialdehyde (MDA), a marker of lipid peroxidation, are increased in the plasma from children with autism (Chauhan et al., 2004). Other studies on erythrocytes (Zoroglu et al., 2004) and urine samples (Ming et al., 2005) have also indicated increased levels of lipid peroxidation markers in autism, thus confirming an increased oxidative stress in autism. Recent studies with the postmortem brain samples from autism and control subjects have provided further evidence on increased oxidative stress in autism. Increased levels of lipid-derived oxidative protein modifications, i.e., carboxyethylpyrrole and iso[4]levuglandin E_2–protein adducts, and heme-oxygenase-1 (an inducible antioxidant enzyme) have been reported in the autistic brain, primarily in the white matter (Evans et al., 2008). Sajdel-Sulkowska et al. (2008) have reported elevated levels of 3-nitrotyrosine (a specific marker for oxidative damage to proteins) in the cerebella of subjects with autism. In addition, we have observed increased lipid peroxidation in cerebellum and temporal cortex of brain in autism (Chauhan et al., 2009). MDA levels were significantly increased by 124% in the cerebellum, and by 256% in the temporal cortex in autism as compared to control subjects.

1.5.2 Lipofuscin in Autism

Lopez-Hurtado and Prieto (2008) revealed a significant increase in the number of lipofuscin-containing cells in the brain of 7- to 14-year-old autistic subjects (by 69% in area 22, 149% in area 39, and 45% in area 44). The increase in the number of lipofuscin-containing cells was paralleled by neuronal loss and glial proliferation. Lipofuscin accumulation is a component of aging (Brunk and Terman 2002a,b; Brunk et al., 1992; Szweda et al., 2003), the neurodegeneration observed in Alzheimer's (Stojanovic et al., 1994) and Parkinson's diseases (Tórsdóttir et al., 1999), developmental syndromes such as Rett syndrome (Jellinger et al., 1988), and autism (Lopez-Hurtado and Prieto, 2008), and such psychiatric disorders as bipolar affective disorder (Yanik et al., 2004) and schizophrenia (Akyol et al., 2002; Herken et al., 2001).

Lipofuscin is an intralysosomal deposit of products of autophagocytosis and degradation of cytoplasmic components, including mitochondria, which cannot be degraded further or exocytosed. Oxidative stress is considered the factor contributing to lipid and protein damage and degradation, resulting in lipofuscin production

and accumulation (Brunk et al., 1992; Sohal and Brunk, 1989). The presence of oxidatively modified proteins and lipids in lipofuscin supports the causative link between enhanced oxidative stress, autophagocytosis, and deposition of products of degradation in the lysosomal pathway and lipofuscin (Brunk and Terman, 2002a,b; Szweda et al., 2003; Terman and Brunk, 2004) and suggests that in autism, abnormal development is associated with early signs of oxidative stress and enhanced degradation and, possibly, turnover of cytoplasmic components.

1.5.3 β-Amyloid Precursor Protein and Intraneuronal Amyloid β in Autism

Sokol et al. (2006) detected signs of overexpression of APP in about 40% of autistic subjects. The levels of secreted APP in plasma in children with severe autistic behavior and aggression were two or more times the levels in children without autism, and up to fourfold more than in children with mild autism. The trend observed in autistic children, with higher levels of secreted β-APP and nonamyloidogenic secreted β-APP, and lower levels of Aβ 1–40 compared to controls, suggests an increased α-secretase pathway in autism (anabolic nonamyloidogenic APP processing). Enzyme-linked immunosorbent assay (ELISA) study of blood plasma from 25 autistic children 2–4 years of age and 25 age-matched control children revealed significantly increased level of secreted amyloid precursor protein alpha (sAPP-α) in 60% of autistic children (Bailey et al., 2008). Western blotting analysis confirmed higher levels of sAPP-α in autistic children.

Amino-terminally truncated intraneuronal amyloid (Aβ) is present in the neurons of control subjects, and the amount of intraneuronal Aβ increases with age (Wegiel et al., 2007). This study of 10 brains of autistic people revealed enhanced intraneuronal accumulation of amino-terminally truncated Aβ in 50% of autistic subjects, including in one 5-year-old child and four adults 20, 23, 52, and 62 years of age. A similar pattern was also found in four examined brains of people with autism and isodicentric chromosome 15 (idic15) (Brown et al., 2008). In idic15, excessive accumulation of intraneuronal Aβ might be related to an extra copy of one of the amyloid precursor protein-binding protein (APBA-2) genes localized on chromosome 15. In many brain regions, Aβ is accumulated in large cytoplasmic granules corresponding to deposits of lipofuscin. Numerous large lipofuscin deposits with very strong Aβ immunoreactivity in the neurons of several children and adults with autism appear to reflect severe metabolic stress affecting all of the neurons in the amygdala, all large neurons in the caudate/putamen, a majority of Purkinje cells, and the neurons in the dentate nucleus and nucleus olivaris, but only about 30%–40% of cortical pyramidal neurons. Accumulation of truncated Aβ appears to be a by-product of enhanced degradation of transmembrane APP. The aggregated intracellular Aβ induces the production of ROS and lipid peroxidation products and ultimately results in leakage of the lysosomal membrane (Glabe, 2001). This process appears to affect many neuronal populations, not only in young and old adults, but also in children diagnosed with autism. A metabolic shift with Aβ accumulation in neurons in these brain areas that are involved in the expression of emotions, stereotypic behaviors, and social deficits, such as the amygdala, hippocampus, some

striatal subdivisions, and cerebellum, may contribute to cellular dysfunction and the clinical expression of autism.

1.6 CLINICOPATHOLOGICAL CORRELATIONS

Studies of clinicopathological correlations cover several domains of functional deficits in people with autism, including (a) speech, language, and verbal and nonverbal communication, (b) social deficits and face perception, (c) sensorimotor deficits, and (d) cognitive deficits.

1.6.1 Speech, Language, and Verbal and Nonverbal Communication

Expressive language function of individuals with autism ranges from complete mutism to verbal fluency. Verbal abilities are often accompanied by errors in word meaning (semantics) or language and communicative deficits in social context (social pragmatics) (Rapin, 1996; Stone et al., 1997; Wetherby et al., 1998). Studies of the language-related neocortex, including Wernicke's area (BA 22, speech recognition), Broca's area (BA 44, speech production) and the gyrus angularis (BA 39, reading) of 7- to 44-year-old autistic and 8- to 56-year-old control individuals, revealed reduced numerical density of neurons by 38% in area 22, and by 24% in area 39 in autistic subjects, as well as an increased numerical density of lipofuscin-containing neurons by 50% in BA 22, and 44% in BA 44. These neuronal changes were paralleled by an increase of numerical density of glial cells in all three examined regions. Lopez-Hurtado and Prieto (2008) hypothesized that structural alteration in one or more of these cortical areas may contribute to the communication impairment observed in autism.

1.6.2 Face Perception

All subjects with ASDs have disturbance of social behavior, including abnormalities in social reciprocity and difficulties in use of eye contact, facial expression, and social motivation. Social functioning includes eye contact, processing of faces, identification of individuals, and monitoring of face expression (Baron-Cohen et al., 1994). Patients with autism reveal deficits in face-processing (Grelotti et al., 2001), perception (Schultz, 2005), and recognition (Joseph and Tanaka, 2003).

The face-processing network includes the visual cortex (BA17), which projects via the inferior occipital gyrus to the fusiform gyrus. Fibers from the fusiform gyrus project to the amygdala, and inferior frontal gyrus and orbital cortex (Fairhall and Ishai, 2007; van Kooten et al., 2008). Functional magnetic resonance imaging (fMRI) identified the fusiform gyrus and other cortical regions as supporting face-processing in control subjects, and hypoactivity of the fusiform gyrus in autistic patients (Bolte et al., 2006; Kanwisher et al., 1999; Pierce et al., 2004). Hypoactivation of the fusiform gyrus is believed to be associated with the failure to make direct eye contact in autism (Dalton et al., 2005). Results of imaging-based fusiform volume estimation are inconsistent. Increased (Waiter et al., 2004) and unchanged (Pierce et al., 2001) volume in both hemispheres and increased fusiform gyrus in the left hemisphere (Herbert et al., 2002) were reported. Morphometric studies of the brain of 7 autistic

and 10 control subjects revealed a reduced number of neurons in layers III, V, and VI, and reduced volume of neuronal soma in layers V and VI in the fusiform gyrus. No alterations in Brodman area 17 in these autistic individuals suggest that the input from the visual cortex to the fusiform gyrus is intact. These results indicate the underdevelopment of connections in the fusiform gyrus that may contribute to abnormal face perception in autism (van Kooten et al., 2008).

Bailey et al. (1998) noted abnormalities in cytoarchitectonic organization and neuronal density in the superior frontal cortex and superior temporal gyrus in autism. Neurons in the superior temporal sulcus are sensitive to the angle of gaze (Perrett et al., 1985). Neurons that are attuned to particular facial expressions were found in the inferior and superior temporal lobes (Hasselmo et al., 1989). Cortical areas responsive to faces, facial expressions, and angle of gaze send direct projections to the amygdala (Stefanacci and Amaral, 2000). Pathological changes in the amygdala may play a central role in the dysfunction seen in autism, including disturbed components of social cognition such as attention to and interpretation of facial expressions. fMRI studies show that judging from the expression of another person's eyes what the other person might be thinking or feeling is associated with activation in the superior temporal gyrus, frontal cortex, and amygdala, whereas in subjects with autism, activation appears in the temporal and frontal cortex but not in the amygdala (Baron-Cohen et al., 1999).

1.6.3 Social Attachment—The Role of the Hypothalamus in Behavioral Deficits

Experimental studies revealed that the hypothalamic nucleus paraventricularis (NPV) and the nucleus supraopticus (NSO), producing oxytocin (OT) and vasopressin (VAS), regulate emotional responses, social attachment, cognitive functions, sleep, and appetite (Barden, 2004; Ehlert et al., 2001; Manaye et al., 2005). OT and VAS are relayed from the human brain into the bloodstream via the posterior pituitary. The presence of receptors for both peptides throughout the forebrain, limbic system, thalamus, brain stem, and spinal cord (Raggenbass, 2001) indicates that hypothalamic neuropeptides modulate the function of many brain regions. Developmental changes in the distribution and expression of receptors suggest that the hypothalamic peptides play a significant role both in brain development and function (Shapiro and Insel, 1989). OT is required for the development of social memory. In OT knockout mice, the loss of social memory could be rescued by OT treatment (Ferguson et al., 2000). VAS is necessary for the formation of social memory and OT for retention of newly formed social memories (Popik and Van Ree, 1992; Popik et al., 1992). OT facilitates the learning of social interactions and the formation of associations that are specifically related to the mother (Nelson and Panksepp, 1996).

The initial product of oxytocin mRNA is a polypeptide containing both nanopeptide OT and neurophysin I, separated by tripeptide glycine-lysine-arginine. The result of enzymatic cleavage are intermediate forms containing 10, 11, or 12 amino acids, collectively referred to as carboxy-extended forms of OT (OT-X), and oxytocin (Gabreels et al., 1998; Gainer et al., 1995; Mitchell et al., 1998; Rao et al., 1992). In 5.8- to 11.5-year-old autistic individuals, reduced plasma OT level, deficits in OT prohormone

processing (increase in OT-X), and an increase in the ratio of C-terminal extended forms to OT were found. In control children, nearly all OT-X is metabolized to OT, whereas in autistic children, the immature OT forms serve as the primary circulating molecule in the absence of or in compensation for OT (Green et al., 2001). However, experimental studies show that OT-X is not an effective agonist at OT-sensitive sites (Mitchell et al., 1998). Deficient conversion of OT-X to OT in autism could be the result of alterations in the level of prohormone convertases associated with genetic defects (Cook, 1998; Szatmari et al., 1998). The identification of four single nucleotide polymorphisms located within the OT receptor gene of 195 Chinese autistic subjects indicates that abnormal modulation of the OT receptor results in autism (Wu et al., 2005). OT and VAS are known to play a role in repetitive behaviors. Patients with ASDs show a significant reduction in repetitive behaviors following OT infusion (Hollander et al., 2003). In about 60% of subjects with autism, abnormal sleep patterns are observed. VAS is involved in the control of circadian rhythmicity (Swaab, 1997). VAS enhances aggressiveness, anxiety, stress levels, and the consolidation of fear memory (Bielsky et al., 2004; Griebel et al., 2002; Landgraf and Neumann, 2004). OT decreases anxiety and stress; facilitates social encounters, maternal care, and the extinction of conditioned avoidance behavior (Bale et al., 2001; Champagne et al., 2001; Windle et al., 1997); reduces activation of the amygdala and modulates fear processing (Kirsch et al., 2005). The presence of abnormal levels of hypothalamic neuropeptides in patients with autism provides strong evidence that an abnormality in OT, VAS and other hypothalamic neuropeptides may have a significant contribution to the behavioral features of autism. However, the morphology and biochemistry of the hypothalamus of autistic subjects remains unknown. The only study of the hypothalamic mammillary body of a 26-year-old autistic man revealed that the cell-packing density was increased by 66% (Bauman and Kemper, 1985).

1.6.4 Sensorimotor Deficits, and Repetitive and Stereotyped Behaviors

In individuals with autism, impairments of gross and fine motor function recognized as hypotonia, limbic apraxia, and motor stereotypes are common findings and are more severe in subjects with lower IQ (Rogers et al., 1996). Hand mannerisms, body rocking, or unusual posturing are reported in 37% to 95% of individuals (Lord, 1995; Rapin, 1996; Rogers et al., 1996). In 42% to 88% of subjects with autism, aberrant sensory processing results in a preoccupation with sensory features of objects, over- or underresponsiveness to environmental stimuli or paradoxical responses to sensory stimuli (Kientz and Dunn, 1997). Sensorimotor deficits may by associated with pathological changes in both the nigrostriatal system (basal ganglia) and the cerebellum (Bailey et al., 1998; Kemper and Bauman, 1998; Ritvo et al., 1986; Saitoh and Courchesne, 1998; Sears et al., 1999). Cerebellar abnormality with a deficit/loss of Purkinje cells (Bailey et al., 1998; Kemper and Bauman, 1993, 1998; Ritvo et al., 1986) has been a common finding. Individuals with autism have been classified as affected by cerebellar hyper- or hypoplasia (Saitoh and Courchesne, 1998). A reduced number of Purkinje cells without significant glial activation and a reduced size of Purkinje cells were noticed in the majority of cerebellar reports (Bailey et al., 1998; Fehlow et al., 1993; Kemper and Bauman, 1993; Lee et al., 2002; Ritvo et al., 1986) in 21 of 29 examined cases (Palmen et al., 2004).

Results of evaluation of the size of the cerebellum using MRI are inconsistent. In several MRI studies, smaller cerebellar hemispheres (Gaffney et al., 1987; Murakami et al., 1989) and vermis (Ciesielski et al., 1997; Courchesne et al., 1988; Hashimoto et al., 1995) were reported. In a study by Piven et al. (1997a), the total cerebellar volume was found to be greater in subjects with autism than in the control group, and the increase was proportional to the increased total brain volume. In the cerebellum, boys with autism had less gray matter, a smaller ratio of gray to white matter, and smaller lobules VI and VII than did controls. Despite the inconsistency of reports characterizing topographic autism-associated vermian hypoplasia (Hashimoto et al., 1993; Kaufmann et al., 2003; Levitt et al., 1999; Piven et al., 1997a; Schaefer et al., 1996), several reports show associations between the size of the vermis and deficits in attention-orienting (Harris et al., 1999; Townsend et al., 1999), stereotypic behavior, and reduced exploration in autism (Pierce and Courchesne, 2001).

The reduced size of the pons, midbrain, and medulla in autism reported by Hashimoto et al. (1992, 1993, 1995) was not confirmed in other studies (Hsu et al., 1991; Piven et al., 1992).

Changes in neurons in the deep cerebellar nuclei were noticed by some authors (Kemper and Bauman, 1998) but not by others (Bailey et al., 1998). Structural MRI shows variable patterns of changes. Volumetry of the cerebellum may show no change, hypoplasia, or hyperplasia. Courchesne et al. (1988) reported selective hypertrophy of lobules VI and VII, but these results were not confirmed in other subjects. In part, the pattern may correspond to the functional status of subjects. In highly functioning subjects with autism, hypoplasia of the cerebellum has not been detected (Holttum et al., 1992).

A decrease in the number of GABAergic Purkinje cells is considered the most consistent neuropathological finding in autism, as it was detected in at least 50% of examined cases (Arin et al., 1991; Bailey et al., 1998). Recent studies indicate that preserved Purkinje cells reveal a 40% decrease in the expression of glutamic acid decarboxylase 67 (GAD67) mRNA in autistic subjects relative to control patients (Yip et al., 2007). Moreover, in autism, the basket cells likely provide increased GABAergic feed-forward inhibition to Purkinje cells. The result may include disruption in the timing of Purkinje cell firing and altered inhibition of the cerebellar nuclei, which could directly affect cerebellocortical output, leading to changes in motor behavior and cognition (Yip et al., 2008).

Repetitive and stereotyped behaviors defined as recurring, nonfunctional activities, or interests that occur regularly and interfere with daily functioning are a defining signs of autism. These behaviors include lower-order repetitive motor behavior, intense circumscribed patterns of interests, and higher-order rituals and compulsions (Gabriels et al., 2005). Several studies implicated the role of basal ganglia and frontostriatal circuitry in the pathophysiology of autism, especially in repetitive and stereotyped behaviors. Increased volume of the basal ganglia was reported in several MRI studies (Herbert et al., 2003; Hollander et al., 2005; Langen et al., 2007; Sears et al., 1999). Sears et al. (1999) and Hollander et al. (2005) observed a positive correlation between caudate volumes and repetitive behavior scores. A significant increase in caudate nucleus volume, disproportional to brain volume, was detected in MRI studies in two independent samples of medication-naive subjects with autism

(21 high-functioning children and adolescents, and 21 typically developing subjects; 21 high-functioning adolescents and young adults, and 21 healthy subjects) (Langen et al., 2007). Our studies showing a significantly smaller size of neurons in the caudate, putamen, and nucleus accumbens, especially in the brains of children 4–7 years of age suggest a developmental delay in the growth of neurons in the basal ganglia of autistic subjects, which may contribute to basal ganglia dysfunction (Wegiel et al., 2008). MRI and postmortem morphometric studies support the hypothesis that developmental abnormalities in frontostriatal circuitry contribute to repetitive and stereotyped behaviors, which are one of three defining symptoms of autism.

1.6.5 Cognitive Deficits

Many individuals with autism demonstrate a particular pattern on intellectual tests that is characteristic of autism. Performance IQ is usually higher than verbal IQ, and block design is the highest subtest, whereas comprehension is usually the lowest (Siegel et al., 1996). Individuals with autism have poorer adaptive function than would be predicted by IQ alone (Volkmar et al., 1993).

Cognitive deficits might be related in part to the memory system and limbic region abnormalities. Reduced volume of both the hippocampal formation and amygdala were noticed in subjects examined by Aylward et al. (1999), but not in populations examined by other researchers (Piven et al., 1998). Neurons in the hippocampus have reduced complexity of dendritic arbors. They are smaller and more densely packed in various portions of the hippocampal formation, entorhinal cortex, medial nuclei of the amygdala, medial septal nucleus, mammillary nuclei, and anterior cingulate gyrus (Bauman and Kemper, 1985; Kemper and Bauman, 1993). Haznedar et al. (1997) observed reduced volume of the anterior cingulate gyrus and decreased positron emission tomography (PET) activity in subjects with autism. Because the cerebellum is involved in a variety of cognitive and affective processes, abnormalities of both the limbic system and the cerebellum may be linked to the core social and communicative deficits in autism.

The caudate nucleus is an integral component of the frontostriatal network involved in cognitive functions (Chow and Cummings, 1999; Voelbel et al., 2006), including learning (Poldrack et al., 1999), short- and long-term memory (Fuh and Wang, 1995), and planning and problem-solving (Mendez et al., 1989; Schmidtke et al., 2002). The increased volume of the caudate observed in autistic children may be indicative of impaired neuronal pruning, contributing to a decrease in executive function (Voelbel et al., 2006).

1.6.6 Epilepsy-Associated Pathology

The 1% prevalence of epilepsy in the general population increases to 8% in DS, 10% in AD (Menendez, 2005; Risse et al., 1990; Velez and Selwa, 2003), and 33% in autism (Tuchman and Rapin, 2002). The interpretation of developmental changes in autism has been challenged by the need to differentiate among lesions that are not associated with epilepsy, that cause epilepsy, and that are produced by epilepsy (Sutula and Pitkanen, 2001). Recent studies support the hypothesis that epilepsy

induces brain alterations that contribute to changes in circuitry, which potentiates the seizure-genic focus (Armstrong, 2005).

Studies of nonautistic subjects indicate that epilepsy-associated pathology includes patchy or laminar neuronal loss and gliosis in the cerebral cortex in one or both hemispheres. In temporal epilepsy, abnormalities were reported in 75% of the specimens examined, and hippocampal sclerosis was found in 50% (Bruton, 1988). Loss of hippocampal neurons correlates with the frequency of tonic-cloning seizures and the total duration of epilepsy (Dam, 1980; Tasch et al., 1999). Loss is accentuated in the CA4 sector and is observed in the granule cell layer in the dentate gyrus. Dispersion of dentate gyrus granular neurons might be a result of seizure-related, disturbed migration of neurons (Bengzon et al., 1997), or epilepsy-enhanced neurogenesis (Ericksson et al., 1998). Ammon horn sclerosis is a progressive lesion that can be induced and propagated by seizures (Armstrong, 2005).

In nearly all cases with hippocampal pathology, changes are also observed in other brain regions. In about 25%, the amygdala, thalamus and mammillary body are affected with neuronal loss. More severe neuronal loss and gliosis in the hippocampus is paralleled by severe neuronal loss and gliosis in the lateral nucleus in the amygdala (Bruton, 1988; Hudson et al., 1993; Thom et al., 1999). Ectopias with more than 8 neurons per 2 sq. mm of white matter occurred in 43% of epileptic patients but in none of the controls (Hardiman et al., 1988). In 45% of severely affected epileptics, significant neuronal loss and astrocytosis spreading out into the overlying molecular layer is observed in the cerebellar cortex. The severity of the cerebellar damage may range from gross atrophy of most or many folia to the restricted neuronal loss in some folia, especially at their basal portion (Gessaga and Urich, 1985).

Central apnea, asphyxia, and pulmonary edema occurring during a seizure (Nashef et al., 1996) as well as life-threatening cardiac arrhythmias during seizures (Earnest et al., 1992; Jallon, 1997; Nashef et al., 1996; Reeves et al., 1996; Saussu et al., 1998) have been suggested as possible causes of sudden unexpected death in epilepsy (Thom et al., 1999).

Enhanced electric activity of neurons and/or increased cell synaptic transmission with enhanced vesicle exocytosis, both in normal and in disease-affected brains are a common cause of modifications of APP processing and Aβ levels. Epilepsy is associated with an elevation of APP expression (Sheng et al., 1994) and occurs in 10 of 11 examined subjects with diffuse nonfibrillar Aβ plaque formation (mean age 47.9 ± 8.8 years of age) (Mackenzie and Miller, 1994; Mackenzie et al., 1996).

1.7 MECHANISMS AFFECTING BRAIN DEVELOPMENT

1.7.1 BDNF AND NEUROTROPHINS IN AUTISM

The neurotrophins, a related family of growth factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins (NT) NT-3 and NT-4/5, have a major role in the survival, growth, and differentiation of neurons (Conner et al., 1997). During typical brain development, only neurons making

the appropriate connections survive and form synapses, whereas neurons that fail to obtain adequate neurotrophins die (Oppenheim, 1991). BDNF is broadly distributed throughout the human central nervous system (CNS) and provides neurotrophic support for many neuronal populations in the cortex, amygdala, hippocampus, and striatum (Murer et al., 2001; Schmidt-Kastner et al., 1996; Tapia-Arancibia et al., 2004). The hypothalamus is the brain structure that contains the highest BDNF protein levels (Katoh-Semba et al., 1997; Nawa et al., 1995; Yan et al., 1997) and BDNF mRNA (Castren et al., 1995; Kawamoto et al., 1996; Yan et al., 1997). In the cerebellum, immunoreactivity was observed in Purkinje cells and the olivary complex of the nuclei (Kawamoto et al., 1996; Murer et al., 2001).

In the basal forebrain of autistic individuals, the level of BDNF was three times higher than in controls (Perry et al., 2001). Miyazaki et al. (2004) observed a higher level of BDNF in the blood samples of young children with autism than in adult control subjects. The mean BDNF levels in sera of children diagnosed with autism and childhood disintegrative disorder were about four times higher than in control children (Connolly et al., 2006). Children with autism and childhood disintegrative disorder have both elevated BDNF levels and levels of autoantibodies against BDNF. Serum BDNF has been shown to be increased after seizures (Binder et al., 2001; Chavko et al., 2002).

1.7.2 Brain Stem and the Role of Serotonin in Brain Development and Clinical Features of Autism

Because 5-hydroxytryptamine (5-HT; serotonin) serves as both a neurotransmitter and an important developmental signal in the brain, dysregulation of the 5-HT system during development may be responsible for many of the abnormalities seen in autism (Whitaker-Azmitia, 2005). In fact, all known chemical inducers of autism including cocaine, thalidomide, valproate, and alcohol modulate 5-HT levels in the brain (Harris et al., 1995; Kramer et al., 1994; Narita et al., 2002; Rathbun and Druse, 1985; Stromland et al., 1994; Williams et al., 2001). A high proportion of children with autism exhibit elevated blood 5-HT levels (hyperserotonemia) and specific alterations in 5-HT biosynthesis. The severity of hyperserotonemia is correlated with the severity of autistic behaviors (Chandana et al., 2005; Chugani et al., 1999; Kuperman et al., 1987). A causal role for serotonergic abnormalities in the etiology of autism is also suggested by studies indicating autism-specific genetic polymorphisms in 5-HT metabolizing enzyme, transporter, or receptor genes (Cohen et al., 2003; Sutcliffe et al., 2005). Also, gender-specific differences in serotonergic regulation during development (Chandana et al., 2005; Chugani et al., 1999), combined with a 52% higher rate of 5-HT biosynthesis in the male than female brain (Nishizawa et al., 1997), and the increased susceptibility of males to early insults imposed by elevated levels of 5-HT (Johns et al., 2002), may contribute to the fourfold higher propensity of males to develop autism compared to females.

As a result of the regulatory role of serotonin affecting the size of neurons, the size of dendritic tree and the number of synapses in innervated cortical and subcortical structures and cerebellum, developmental abnormalities in the serotonergic

system may contribute to structural and functional changes in target brain regions and structures. Virtually all regions of the brain receive serotonergic afferents from raphe system neurons. The rostral raphe nuclei form ascending pathways of axons mainly to the forebrain. The caudal raphe system innervates the lower brain stem and the spinal cord (Aitken and Törk, 1988; Lidov and Molliver, 1982). The functions of serotonin are mediated by 14 subtypes of 5-HT receptors in the nervous system (Hoyer et al., 1994; Martin and Humphrey, 1994; Saudou and Hen, 1994a,b). The serotonin$_{2A}$ (5-HT$_{2A}$) receptor is known to be one of the major subtypes and is associated with psychological and mental events (Roth, 1994). The 5-HT$_{2A}$ receptor plays a role in facilitating the formation and maintenance of synapses (Niitsu et al., 1995). Staining for 5-HT$_{2A}$ shows the entire somata and dendritic tree of Purkinje cells in a rat cerebellum (Maeshima et al., 1998). In vitro studies have shown that that 5-HT inhibits the growth and arborization of Purkinje cell dendrites through 5-HT$_{2A}$ receptors and stimulates them through the 5-HT$_{1A}$ receptor (Kondoh et al., 2004). 5-HT promotes the formation of synapses in developing and mature brain and spinal cord (Chen et al., 1997; Niitsu et al., 1995; Okado et al., 1993), and this process is mediated by the 5-HT$_{2A}$ receptor in the spinal cord (Niitsu et al., 1995). Biochemical studies support the hypothesis that developmental defects of the raphe nuclei may make a major contribution to the structural and functional defects of cortical and subcortical structures. However, raphe nuclei have not yet been examined in autistic subjects.

1.8 CLOSING REMARKS

The detected brain structure–specific patterns of structural aberrations in a majority of examined anatomic subdivisions in autism brain may contribute to deficits in expression of emotions, processing of social stimuli, learning of social behaviors, verbal and nonverbal communication, and stereotypic behaviors. Pathological acceleration of brain growth and immaturity of neurons and neuronal networks in early childhood indicate that (a) a significant portion of structural/functional defects starts in early infancy and (b) causative factors dysregulate the mechanisms controlling brain/neuron development. The deceleration of brain growth in the second year of life and the increase of neuronal size in late childhood/adulthood suggests delayed activation of correcting mechanisms. However, the delayed correction of brain and neuronal size does not result in functional recovery. Analysis of the detected pattern of abnormal brain development in autism indicates that early diagnosis and early treatment may prevent or reduce developmental delay, reduce/eliminate secondary structural and functional changes, and improve clinical status throughout the life span.

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ORIGINAL PAPER

The neuropathology of autism: defects of neurogenesis and neuronal migration, and dysplastic changes

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Abstract Autism is characterized by a broad spectrum of clinical manifestations including qualitative impairments in social interactions and communication, and repetitive and stereotyped patterns of behavior. Abnormal acceleration of brain growth in early childhood, signs of slower growth of neurons, and minicolumn developmental abnormalities suggest multiregional alterations. The aim of this study was to detect the patterns of focal qualitative developmental defects and to identify brain regions that are prone to developmental alterations in autism. Formalin-fixed brain hemispheres of 13 autistic (4–60 years of age) and 14 age-matched control subjects were embedded in celloidin and cut into 200-μm-thick coronal sections, which were stained with cresyl violet and used for neuropathological evaluation. Thickening of the subependymal cell layer in two brains and subependymal nodular dysplasia in one brain is indicative of active neurogenesis in two autistic children. Subcortical, periventricular, hippocampal and cerebellar heterotopias detected in the brains of four autistic subjects (31%) reflect abnormal neuronal migration. Multifocal cerebral dysplasia resulted in local distortion of the cyto-architecture of the neocortex in four brains (31%), of the entorhinal cortex in two brains (15%), of the cornu Ammonis in four brains and of the dentate gyrus in two brains. Cerebellar flocculonodular dysplasia detected in six subjects (46%), focal dysplasia in the vermis in one case, and hypoplasia in one subject indicate local failure of cerebellar development in 62% of autistic subjects. Detection of flocculonodular dysplasia in only one control subject and of a broad spectrum of focal qualitative neuropathological developmental changes in 12 of 13 examined brains of autistic subjects (92%) reflects multiregional dysregulation

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of neurogenesis, neuronal migration and maturation in autism, which may contribute to the heterogeneity of the clinical phenotype.

Keywords Autism · Developmental neuropathology · Subependymal nodular dysplasia · Heterotopia · Dysplasia

Introduction

Autism is characterized by a broad spectrum of clinical manifestations, including (a) qualitative impairments in reciprocal social interactions, (b) qualitative impairments in verbal and nonverbal communication, (c) restricted repetitive and stereotyped patterns of behavior, interests and activities and (d) onset prior to the age of 3 years [1]. In most cases, the etiology is unknown, and patients are diagnosed with idiopathic or non-syndromic autism [10, 43]. About 70% of individuals with idiopathic autism have essential autism, defined by the absence of physical abnormalities, but in 30%, complex autism with dysmorphic features such as microcephaly and/or a structural brain malformation is diagnosed [79]. In 5–10% of cases, autism is diagnosed in association with other disorders such as fragile X syndrome, Rett syndrome, Down syndrome, and tuberous sclerosis [94, 105]. Intellectual impairments, defined as intelligence quotient (IQ) scores less than 70, were reported in 44.6% of children diagnosed with autism [28]. Epilepsy is observed in up to 33% of individuals with autism [106].

The phenotypic heterogeneity is a major obstacle in all areas of autism research [83] and may be the result of a contribution of non-overlapping gene effects. The genetic fractionation of social impairment, communication difficulties and rigid and repetitive behaviors suggests that different features of autism are caused by different genes associated with different brain regions and are related to different cognitive impairments and functional abnormalities [48].

In spite of the broad spectrum of clinical manifestations and striking inter-individual differences, studies of thousands of children have resulted in establishing the clinical diagnostic criteria of pervasive developmental disorder [1]; however, corresponding neuropathological diagnostic criteria do not exist. One of the reasons for the disproportionate progress in clinical and neuropathological studies is the limited tissue resources available for postmortem studies. Between 1980 and 2003, only 58 brains of individuals with autism were examined [85]. Due to the diversity of research aims, of protocols for tissue preservation and of methods of sampling and examination, and the small number of brains examined in an individual project, the pattern of neuropathological changes emerging from these studies remains incomplete and inconsistent.

The hypothesis that autism is associated with neuropathological changes was explored in the first reports published between 1980 and 1993 [7, 21, 22, 27, 42, 50, 51, 82, 90]. Since then, implementation of broader diagnostic terms such as Autism Spectrum Disorder (ASD), examination of larger cohorts, applications of stereology, and functional and structural magnetic resonance imaging (MRI) have resulted in the detection of several major types of pathology, most likely contributing to the clinical phenotype. An emerging concept of autism-related brain pathology integrates evidence of (a) abnormal acceleration of brain growth in early childhood [89], (b) minicolumn pathology [13, 14], (c) curtailed neuronal development [7, 108] and brain structure-specific delays of neuronal growth [111] with indications of abnormalities in brain cytoarchitecture [4, 7], metabolic modifications with abnormal amyloid protein precursor (APP) processing [5, 101], enhanced oxidative stress [17] and enhanced turnover of cell organelles with pigment accumulation and glial activation [68].

In spite of the conceptual limitations, "localizing" models are still the main approach to the identification of pathological changes as a component of the networks' structural and functional abnormalities [81]. We hypothesize that dysregulation of neurogenesis, neuronal migration and maturation is also reflected in qualitative, focal, developmental alterations of brain microarchitecture. The aim of this study is to detect the pattern of focal, qualitative, developmental defects in autism brain, including their type, topography and severity, and to identify the structures and brain regions that are prone to developmental alterations in autism.

Materials and methods

The autistic cohort study consisted of 13 subjects (4–62 years of age), including 9 males (69%) and 4 females (31%), while the control cohort consisted of 14 subjects (4–64 years of age), including 9 males and 5 females (Table 1).

Clinical and genetic characteristics of the autistic subjects

The source of our clinical data was the medical records of the autistic subjects, which consisted of psychological, behavioral, neurological and psychiatric evaluation reports. All of the records were obtained after the subjects' deaths. The Autism Diagnostic Interview-Revised (ADI-R) was administered to each donor family as a standardized assessment tool in order to confirm the diagnosis on a postmortem basis. Inclusion of the subject in this study was based on a summary of scores of four domains:

Table 1 Material examined

#	Group	Brain bank #	Sex	Age (years)	Cause of death	PMI (h)	H	Brain weight (g)
1	A	IBR425-02	M	4	Drowning	30	R	1,280
2	A	UMB-1627	F	5	Traumatic multiple injuries	13.2	R	1,390
3	A	B-6403	M	7	Drowning	25	R	1,610
4	A	B-5666	M	8	Rhabdomyosarcoma	22.2	R	1,570
5	A	B-5342	F	11	Seizure-related drowning	12.9	L	1,460
6	A	B-5535	M	13	Seizure-related	8	L	1,470
7	A	B-6115	F	17	Cardiac arrest related to cardiomyopathy	25	L	1,580
8	A	UMB-1638	F	21	Seizure-related respiratory failure	50	R	1,108
9	A	B-6337	M	22	Seizure-related	25	R	1,375
10	A	IBR93-01	M	23	Status epilepticus-related respiratory failure	14	R	1,610
11	A	B-6212	M	36	Cardiac arrest	24	R	1,480
12	A	B-6276	M	56	Cardiac arrest	3.35	R	1,570
13	A	B-7090	M	60	Pancreatic cancer	26.5	R	1,210
1	C	B-6736	F	4	Acute bronchopneumonia	17	R	1,530
2	C	UMB-1499	F	4	Lymphocytic myocarditis	21	R	1,222
3	C	UMB-4898	M	7	Drowning	12	R	1,240
4	C	UMB-1708	F	8	Traumatic multiple injuries	20	R	1,222
5	C	BTB-3638	M	14	Electrocution	20	R	1,464
6	C	UMB-1843	F	15	Traumatic multiple injuries	9	R	1,250
7	C	UMB-1846	F	20	Traumatic multiple injuries	9	R	1,340
8	C	UMB-1646	M	23	Ruptured spleen	6	R	1,520
9	C	UMB-4543	M	29	Traumatic multiple injuries	13	R	1,514
10	C	UMB-1576	M	32	Traumatic compressional asphyxia	24	R	1,364
11	C	BTB-3899	M	48	Atherosclerotic heart disease	24	L	1,412
12	C	IBR252-02	M	51	Myocardial infarct	18	L	1,450
13	C	BTB-3983	M	52	Heart atherosclerosis	13	R	1,430
14	C	B-6874	M	64	Cardiac arrest	28	R	1,250

PMI postmortem interval, *H* hemisphere, *R* right, *L* left

(a) qualitative abnormalities in reciprocal social interaction; (b) qualitative abnormalities in verbal and nonverbal communication; (c) restricted, repetitive and stereotyped patterns of behavior; and (d) abnormality of development evident at or before 36 months [69]. All 13 autistic subjects met ADI-R criteria for autism. For some subjects, the intellectual evaluation was available and was based on the Wechsler Intelligence Scale for Children III and the Woodcock-Johnson Tests of Achievement-Revised (Table 2). Eight subjects were diagnosed with intellectual disability, usually in the range from mild to severe (61%). Six of 13 autistic subjects had seizures (46%). In five cases, the age of onset of seizures was from 14 months to 5 years of age. A 23-year-old autistic male had only one seizure, which was reported as the cause of his death. In one child, an abnormal EEG was detected, but without seizures.

Several forms of challenging behaviors and behavioral disorders were noted, including self-injurious behavior (six cases, 46%), aggression (four cases, 31%), hyperactivity (three cases, 23%), obsessive compulsive disorder (two cases, 12%) and depression and mania (a single case of each).

For three of the 13 autistic subjects, the list of high-confidence copy number variations identified both by quantiSNAP and Partek HMM computational algorithm was posted on the ATP portal by Drs. Steve Scherer and Richard Wintle from The Center for Applied Genomics, Toronto. The copy number variations detected in the three autistic subjects do not differ from those commonly observed [75], except for the loss of 25,505 kb within Neuropeptide S Receptor 1 (NPSR1) gene at 7p15–p14 detected in a 22-year-old autistic male (B-6337). NPSR1 has not been linked to autism in the genomic reports [103, 112]; however, an association of NPSR1 copy number variation with allergies has been reported [11] that might be linked to the patient's history of allergies.

Originally, 38 brains, including 20 brains of autistic and 18 brains of control subjects, were assigned to this project. However, application of the clinical and neuropathological

Table 2 Behavioral and neurological signs, and the type and topography of developmental abnormalities

Brain bank #	Psychiatric disorders and neurological symptoms	Mental retardation (MR)	Seizures age of onset	Type and topography of developmental abnormalities
IBR425-02	Hyperactivity. Tantrums. Self-injurious behavior	–	–	No changes
UMB-1627	Aggression	–	–	Focal neuronal heterotopia in white matter of the anterior cingulate gyrus
B-6403	–	–	14 months	Subependymal nodular dysplasia in the wall of the occipital horn of the lateral ventricle. Two periventricular nodular heterotopias (2 and 4 mm in diameter) near the frontal horn of the lateral ventricle. Tuber-like expansion of the tail of caudate nucleus into the lumen of the ventricle. Flocculonodular dysplasia
B-5666	–	–	Abnormal EEG; no seizures	Cortical dysplasia in the middle and inferior temporal gyri with focal dyslamination, clustering of dystrophic neurons and severe local neuronal deficits. Several focal dysplastic changes within CA. Flocculonodular dysplasia affecting almost entire lobe
B-5342	Pervasive developmental disorder. Hyperlexia	Mild MR	4.5 months	Focal cortical dysplasia. Dysplasia of the granule layer of the dentate gyrus. Subcortical heterotopia in the inferior frontal gyrus. Heterotopia in vermis and in cerebellar white matter
B-5535	Hyperactivity. Self-injurious behavior including head-banging	Moderate to severe MR	2 years	Thickening of the subependymal cell layer. Focal dysplasia within CA1 pyramidal layer with neuronal deficit, abnormal neuron morphology and spatial orientation. Multifocal dysplasia of the dentate gyrus with distortion of the shape of granule and molecular cell layers. Focal dysplasia within vermis
B-6115	Sensory integration disorder	–	–	Flocculonodular dysplasia affecting the majority of lobe volume. Cortical angioma
UMB-1638	ADHD	Moderate MR	5 years	Focal dysplasia within CA1 with diffuse neuronal deficit but without glial activation
B-6337	Obsessive compulsive disorder. Mania. Tourette syndrome. Self-injurious behavior	MR	–	Minor focal flocculonodular dysplasia
IBR93-01	Hyperactivity. Aggressive and self-injurious behavior	Severe MR	23 years	Focal dysplasia within islands in the entorhinal cortex. Pineal gland cysts
B-6212	Obsessive compulsive disorder. Depression, aggression, and anxiety	Severe MR	–	Several areas of focal cortical dysplasia within frontal cortex and insula with local loss of vertical and horizontal organization. Merger of ventral portion of the claustrum with insula. Flocculonodular dysplasia
B-6276	Aggression and self-injurious behavior, anxiety and agitation	Moderate MR	–	Focal dysplasia within CA1 sector with focal neuronal deficit. Heterotopia within stratum oriens. Flocculonodular dysplasia affecting approximately 70% of the lobe
B-7090	Disturbed movement coordination (walking like drunk)	MR	3 years	Three focal dysplasias in the frontal cortex. Dysplasia of layers 1–3 in the entorhinal cortex with missing numerous islands of the stellate neurons. Severe hypoplasia of cerebellar lobes 1–4. Reduced convolutions within dentate nucleus

Developmental abnormalities in brains of autistic subjects

exclusion criteria reduced the size of the cohort to 27 brains. Based on the results of the ADI-R, two cases were excluded, including one case diagnosed with atypical autism, and one that did not meet ADI-R criteria. Based on postmortem evaluation, five more autistic cases were excluded: one due to severe postmortem autolytic changes, three due to severe global hypoxic encephalopathy related to the mechanism of death, and one due to multiple microinfarcts. Moreover, four brains of control subjects were disqualified due to severe postmortem autolysis. In all these brains, neuronal loss, changes of neuronal size and shape, and gliosis were so severe that they masked and distorted the qualitative and quantitative characteristics of the developmental alterations associated with autism.

Brain tissue preservation

Brains of 13 autistic and 14 age-matched control subjects were examined by postmortem MRI and neuropathologically. The postmortem interval (PMI) varied, ranging from 6 to 27.8 h in the control group (16 h on average; SD 6 h) and from 8 to 30 h in the autistic group (20 h on average; SD 12 h). The median PMI was 15 h.

The brain hemispheres were removed using standard techniques, exercising extra care to avoid damaging the brain tissue. The brain was weighed in the fresh state. The fresh brain was sagittally cut through the corpus callosum and brainstem. Half of the brain was fixed in 10% buffered formalin. Following at least 3 weeks of fixation, the brain hemisphere was scanned using MRI. The aim of the MRI application was to determine the type of developmental changes detectable by MRI and to microscopically characterize MRI findings. All brains within this project were scanned (L.A.S.L.) using a standardized protocol (established and implemented for this and for other postmortem MRI studies by L.A.S.L. and M.L.). MRI scans were acquired on a 1.5 T GE Signa Imager (General Electric, Milwaukee, USA). The research scan consisted of a 124-slice T1-weighted fast gradient echo acquired in a coronal orientation perpendicular to the long axis of the hippocampus with a 1.5-mm slice thickness, which encompassed the entire brain hemisphere without gaps or wrap artifacts (FOV = 25 cm; NEX = 1; matrix = 256 × 192; TR = 35 ms; FA = 60°). All file names were assigned sequential code numbers, and demographic information was removed from image headers [9]. MRI scans were first screened in a diagnosis–blind manner, and the brains with abnormalities were re-evaluated by both radiologists and neuropathologists to determine the topography, type, and size of lesions detected with both methods.

The brain hemisphere was fixed with 10% buffered formalin. Formalin was washed out from the tissue during an overnight tap water rinsing. Brains were dehydrated using a series of increasing ethyl alcohol concentrations (50% ethanol 3 days; 70% ethanol 4 days; 80% ethanol 3 days; 95% ethanol 4 days). The brain hemisphere was embedded in 8% celloidin [53]. During hardening, celloidin blocks were exposed to chloroform vapors for approximately 2.5 weeks, and celloidin blocks were then stored in 70% ethanol. For sectioning, the block was attached to the block holder with 10–15 ml of 8% celloidin. To fasten adhesion of the block to the holder, the block with the holder attached was immersed in 70% ethanol overnight. Serial 200-μm-thick sections were separated with filter paper and stored in 70% ethanol. For the four control and four brains of autistic subjects, alternative series of 200- and 50-μm-thick sections were preserved. To ensure the same probability of detection of changes in each case, every 200-μm-thick section, with a distance 1.2 mm, was used in this project. Sections were washed in water for 2–3 h, stained with cresyl violet (CV) and mounted with Acrytol.

One neuropathologist (I.K.) examined, in a blind-to-diagnosis fashion, on average 120 hemispheric CV-stained sections per case with a 1.2-mm distance between sections. Two-step screening included examination at low magnification (28×) using Zeiss DL2 Documator and microscopic examination using objective lenses from 5× to 100×. Two other neuropathologists (T.W. and J.W.) examined all histological slides for which pathology was detected during the primary screening. The defects of neurogenesis, neuronal migration, and dysplastic changes that they detected were summarized in this report.

Tissue acquisition for this program project is based on individual tissue transfer agreements between the program project's principal investigator and several tissue banks: (a) the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, (b) the Harvard Brain Tissue Resource Center and (c) the Brain Bank for Developmental Disabilities and Aging of the NYS Institute for Basic Research in Developmental Disabilities. Each brain hemisphere number given by the institution that received the donation was used as the only identifier of clinical records and tissue samples. Brain Bank identification of tissue samples is listed in Tables 1 and 2 to keep non-overlapping records of results of examination of brains in different projects and research groups. The Institutional Review Board of the New York State Institute for Basic Research in Developmental Disabilities approved the methods applied in this study.

Results

Neuropathological evaluation of serial coronal hemispheric sections from the cerebral and cerebellar hemispheres of

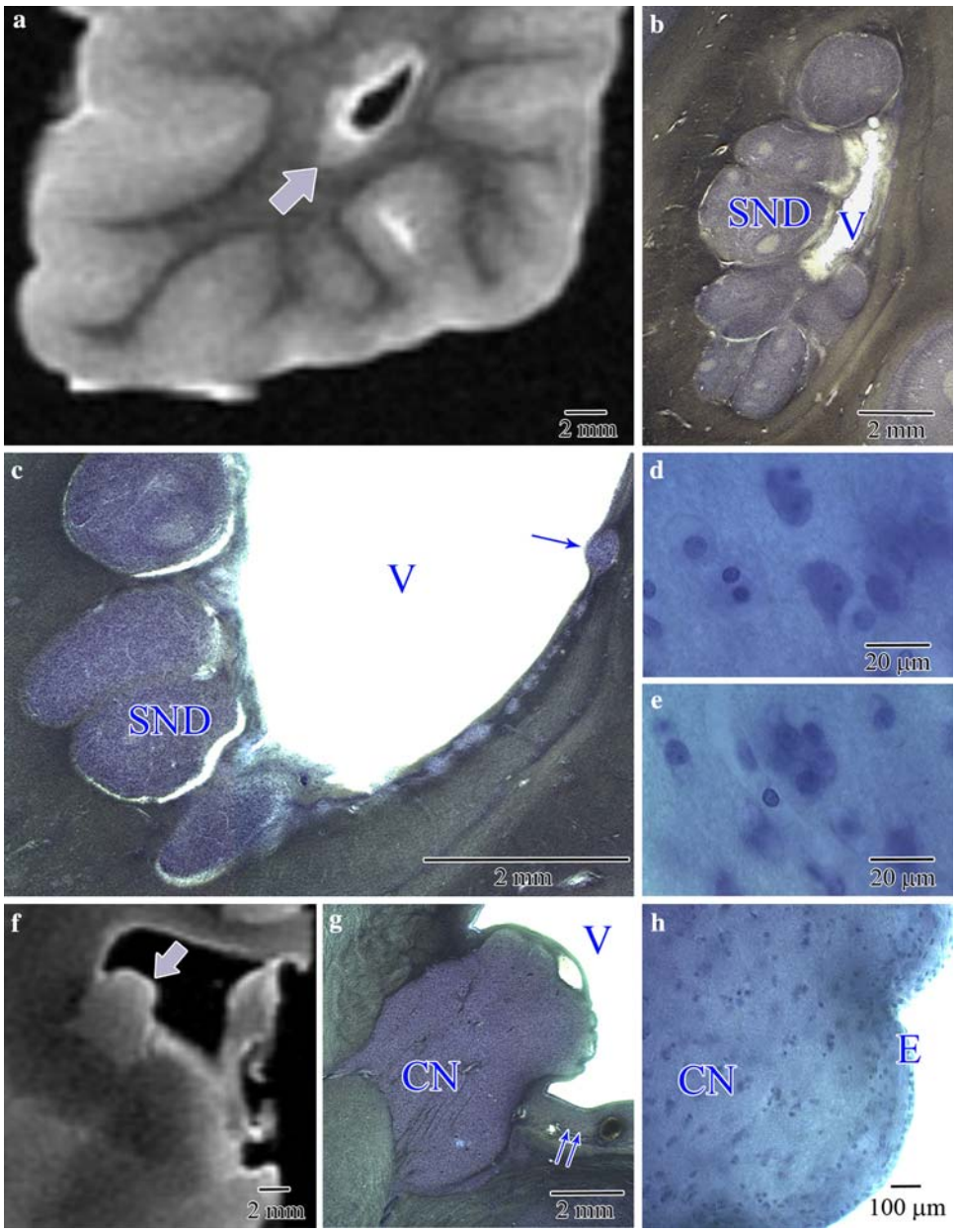


Fig. 1 Nodules in the wall of the lateral ventricle detected in postmortem MRI (**a**) in the brain of a 7-year-old male diagnosed with autism (B-6403) revealed features of subependymal nodular dysplasia (SND; **b**) in examination of CV-stained sections. **c** Numerous large and small nodules (*arrow*) dispersed within subependymal cell layer. They contained a few pyramidal-like neurons (**d**) and numerous poorly differentiated cells (**e**). Tuber-like expansion of the caudate nucleus (*arrow*) into the ventricle lumen is shown in MRI (**f**) and in CV-stained section (**g**). **g** A thick subependymal cell layer above and below (*two arrows*) the caudate nucleus (*CN*), and the absence of matrix in the tuber-like area. Under ependymal (*E*) cap of the caudate nucleus (*CN*) tuber-like expansion, small poorly differentiated neurons are present (**h**)

13 autistic and 14 control subjects revealed more details characterizing the topography and severity of changes than did standard sampling of brains for routine neuropathological evaluation. A broad range of changes was found. Developmental abnormalities included subependymal nodular dysplasia, heterotopia and very common dysplastic changes within the neo- and archicortex, hippocampus and cerebellum in 12 of 13 examined brains of the autistic subjects (92%) (Table 2). The general result of these developmental defects was a multifocal disorganization of gray and white matter. The developmental pathology observed in control brains was limited to one cerebellar dysplasia.

Alterations of the subependymal cell layer and subependymal nodular dysplasia

In two autistic subjects, there was a several-fold local increase in the thickness of the subependymal cell layer. Numerous subependymal nodules were found within a pathologically thickened subependymal cell layer, in the wall of the occipital horn of the lateral ventricle of a 7-year-old male, which reflects a subependymal nodular dysplasia (Fig. 1a–e). Nodules occupied 13.3 mm of the caudal portion of the occipital horn of the lateral ventricle. The diameter of round/oval nodules varied in size from 285 to 3,310 µm. While the smallest nodules were dispersed

within the subependymal cell layer, the large nodules expanded partially into the white matter, and partially into the lumen of the ventricles and were detectable on MRI (Fig. 1a) and CV-stained histological sections (Fig. 1b, c). The effect was narrowing of the ventricle and the tuberous appearance of the ventricular wall. There were large tubers that contained dysplastic neurons with a partially modified morphology of pyramidal, multipolar or bipolar large neurons (Fig. 1d) and irregularly shaped medium and small neurons. Neurons in the small nodules were small and poorly differentiated (Fig. 1e). In the large nodules, several hypocellular areas were observed. The nodules were free of oval or polygonal giant cells or ballooned glial cells, as well as signs of calcification.

In the brain with the subependymal nodular dysplasia, an abnormal tuberous expansion of the caudate nucleus was detected on MRI (Fig. 1f) and in histological sections (Fig. 1g, h). Only the ependyma separated the tuber-like expansion of the caudate from the ventricle lumen. The very thick subependymal cell layer that was present close to the caudate was substituted by loosely arranged and poorly differentiated neurons in the affected area.

Heterotopia

Heterotopias were found in the brains of four autistic subjects and no control subjects. The topography of the lesions was different in each case. Subcortical heterotopias were detected in the white matter of the anterior cingulate gyrus of a 5-year-old (Fig. 2a, b) and in the inferior frontal gyrus in an 11-year-old subject. Periventricular heterotopias were detected near the wall of lateral ventricle in 7-year old subject (Fig. 2c, d). A single heterotopia was noted in the stratum oriens of the hippocampus. In the cerebellum of the 11-year-old subject, heterotopias were detected in the vermis and the cerebellar white matter close to the dentate nucleus (Fig. 2f–h). These defects of migration were observed in two brains as a single aggregate of gray matter, in one brain as two aggregates and in one brain as three lesions measuring from 1 to 3 mm in diameter. Subcortical and periventricular heterotopias comprised poorly differentiated oval or multipolar neurons without spatial orientation (Fig. 2a) or had a distorted laminar organization (Fig. 2e). Cerebellar heterotopias had a distorted morphology of the granule and molecular layers with a few dispersed Purkinje cells (Fig. 2g, h).

Dysplasia within neocortex and archicortex, hippocampus and cerebellum

The multifocal neocortical dysplasia detected in four brains of autistic subjects (31%) was associated with a local loss of vertical and horizontal organization of the neocortex, formation of abnormal layers, loss of orientation of neurons (Fig. 3a, b) and thickening of the affected portion of the cortical ribbon. A focal dysplasia in the entorhinal cortex, observed mainly in the second layer with a local absence of islands and/or reduced number of neurons, was found in the 23-year-old and the 60-year-old autistic subjects (15%) (Fig. 3c, d). A lack of giant multinuclear neurons and large, ballooned glial cells typical of focal cortical dysplasia indicated that the observed developmental changes in neocortex and archicortex reflect a more subtle cortical malformation, classified usually as focal cortical microdysgenesis.

Two types of changes were observed in the dentate gyrus. An abnormal migration of granule neurons into the molecular layer resulted in the formation of an additional fragmentary granule cell layer (Fig. 3e). In other areas, granule cells formed irregular circles and loops (Fig. 3f).

In the CA1 sector of a 13-year-old male, several areas of dysplastic changes with a significant deficit of pyramidal neurons without gliosis were found (Fig. 3g). In affected areas, the size and shape of neurons varied over a wide range. Pyramidal neurons were very rare, whereas small irregular or oval-shaped, poorly differentiated neurons prevailed (Fig. 3h). In the dysplastic area in the CA1 sector of the 56-year-old autistic subject, an opposite trend was present, with thickening of the pyramidal layer and an increased packing of dysplastic neurons (not shown).

The most common developmental abnormality within the cerebellum was dysplasia, which was detected in seven autistic subjects (54%) and in the cerebellum of one control subject.

Flocculonodular dysplasia (Fig. 4a, b), usually affecting the entire nodule, was found in the cerebellum in six autistic subjects (46%). In the dysplastic areas, a thin granule layer formed the labyrinth, which was mixed with irregular islands of the molecular layer. Clusters of granule cells and a few Purkinje cells were dispersed within the distorted molecular layer. The only developmental abnormality detected in the control group was flocculonodular dysplasia in the cerebellum of the 51-year-old control subject (not shown). Local cortical dysplasia was also detected within the vermis of the 13-year-old autistic male. In the affected area, the cytoarchitecture of the molecular and granule layers and the Purkinje cells was completely disorganized (Fig. 4c, d).

In the cerebellum of the 60-year-old autistic male, severe hypoplasia affected lobes 1–4 (Fig. 4e). The thickness of the molecular and granular layer was decreased by almost 50% in comparison to that of the non-affected areas (Fig. 4e, f). The number of Purkinje cells was significantly reduced in the hypoplastic area. Hypoplastic changes within the portion of cerebellar cortex were observed,

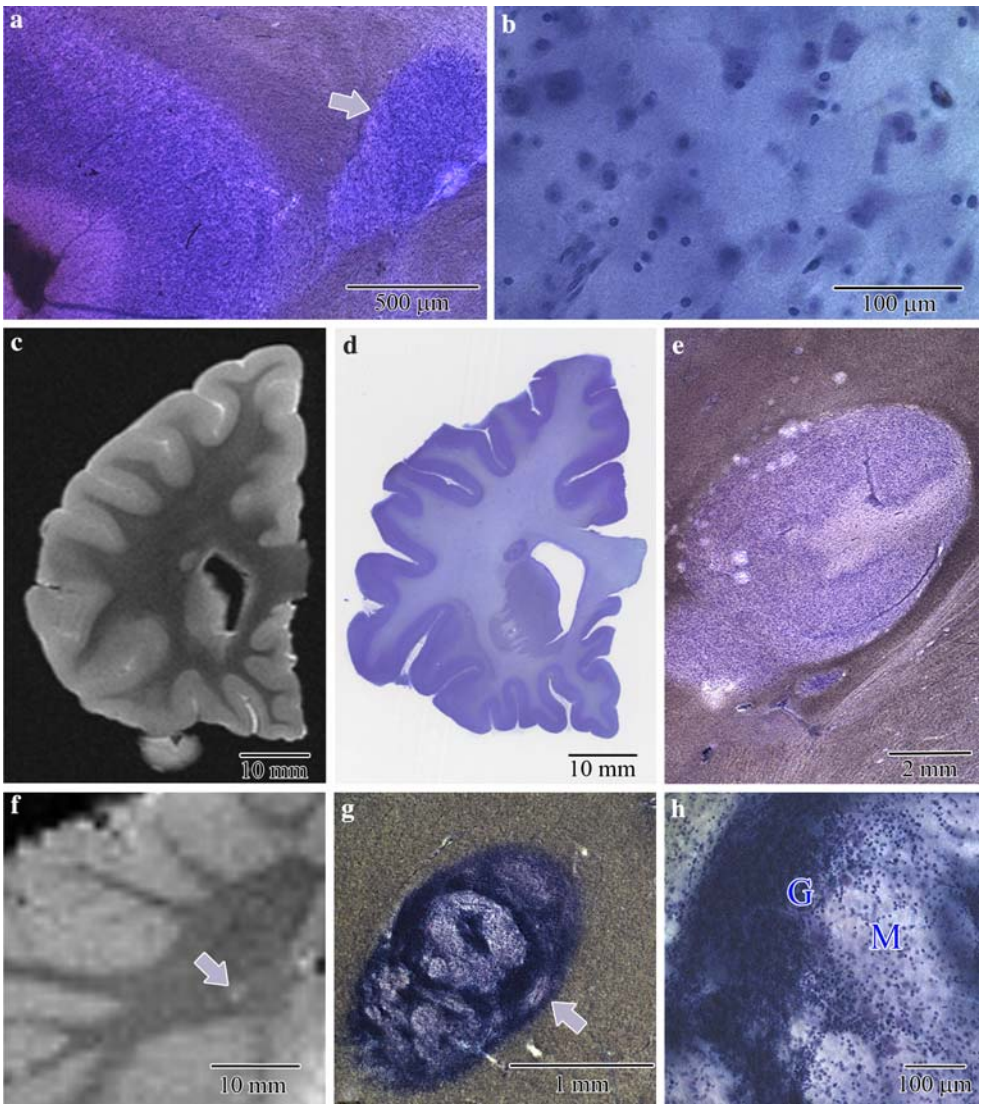


Fig. 2 Large subcortical heterotopia within anterior cingulate gyrus in a 5-year-old autistic child (UMB-1627) (**a**) contained dysplastic neurons without spatial orientation (**b**). Periventricular heterotopia near the frontal horn of the lateral ventricle (**c** MRI, **d**, **e** CV-stained section) shows a structure resembling molecular, granule and pyramidal layers in a 7-year-old autistic subject (B-6403). MRI (**f**), low (**g**) and large (**h**) magnification of heterotopia (*arrow*) with dysplastic granule (*G*) and molecular layer (*M*) detected within cerebellar white matter in an 11-year-old autistic subject (B-5342)

together with a significantly reduced convolution of the dentate nucleus (Fig. 4g).

Discussion

This neuropathological study revealed a broad spectrum of focal developmental abnormalities and pre- and perinatally acquired lesions in 92% of the brains of autistic subjects and striking inter-individual differences in the type and topography of changes. Evidence that different features of autism are caused by different genes associated with different brain regions [48] suggests a link between regional developmental alterations in the brain and different components of the autistic phenotype.

Altered neurogenesis in autism

Increased brain mass in autistic children and some autistic adults [89], increase in the numerical density of neurons [13, 14], reduced size of neurons [7] and brain structure-specific delay of neuronal growth [111] indicate alterations in neuronal and brain growth in autistic individuals. The subventricular zone of the lateral ventricles [26] and the dentate gyrus [33] are active sites of neurogenesis in adult humans. Several of our findings support the hypothesis of

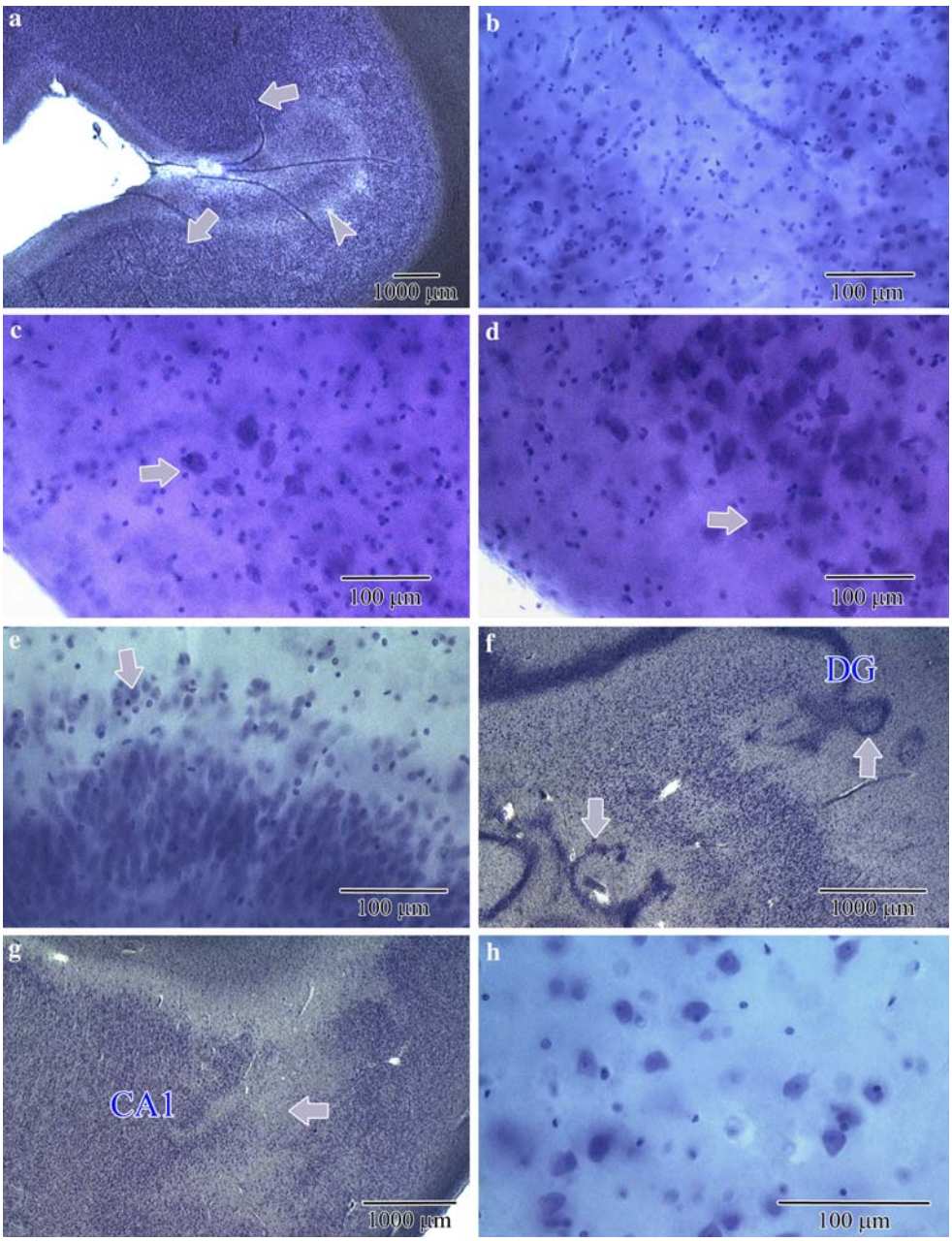


Fig. 3 Dysplastic changes within neocortex (**a**, **b**), entorhinal cortex (**c**, **d**), dentate gyrus (**e**, **f**) and the cornu Ammonis (**g**, **h**). Focal dysplasia in frontal cortex with loss of vertical and horizontal cytoarchitecture (*two arrows*) and abnormal (*arrowhead*) laminar organization (**a**). Dysplastic neurons within affected area (B-6212) (**b**). Microdysgenesis within the entorhinal cortex with deficit of stellate neurons in the islands (**c**) and normal morphology of islands in adjacent cortex (**d**) in 60-year-old autistic subject (B-7090). Microdysgenesis of the dentate gyrus with dispersion of granule cells within the molecular layer (**e**, *arrow*) and distortion of the granule cell layer shape (**f**, *arrows*) in 13-year-old autistic male (B-5535). CA1 sector microdysgenesis with local deficit of pyramidal neurons (**g**, *arrow*) without markers of gliosis but with signs of poor differentiation of dysplastic abnormally arranged neurons (**h**) in 13-year-old autistic subject (B-5535)

altered neurogenesis in autistic subjects. The increased thickness of the subependymal cell layer, subependymal nodular dysplasia, abnormal growth of the dentate nucleus and dysplasia of the granule layer in the dentate gyrus, detected in this study, appear to be signs of abnormal neurogenesis in the brains of three autistic subjects. Subependymal nodules were reported in approximately 80% of patients with tuberous sclerosis, a disorder that is highly associated with epilepsy, autism and mental retardation [73]. Tuberous sclerosis nodules were detected in one fetus [12], suggesting that fetal development of subependymal nodules can lead to the early onset of epilepsy

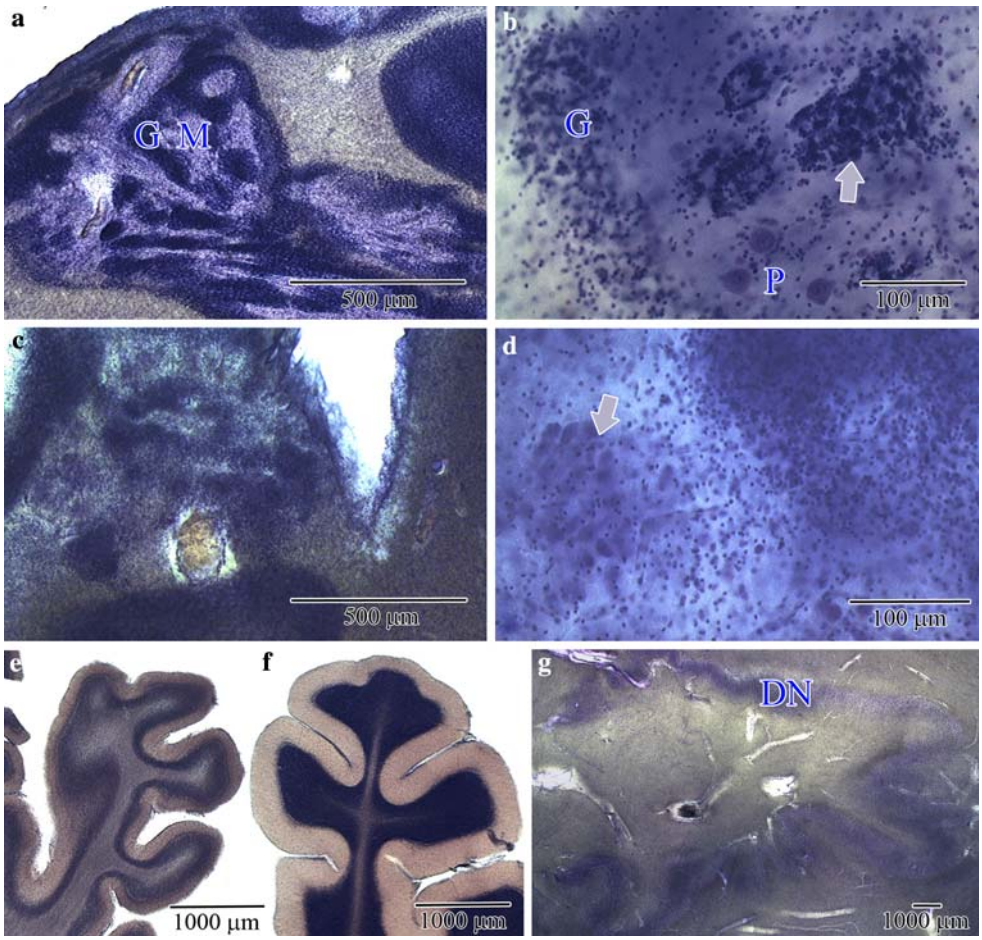


Fig. 4 Flocculonodular dysplasia in cerebellum of 56-year-old autistic subject (B-6276) (**a**) with thin irregular granule (*G*) and molecular (*M*) layer. **b** Dysplastic granule layer (*G*), ectopic granule cells (*arrow*) in the molecular layer, and loosely dispersed Purkinje cells (*P*) (B-6276). Cortical dysplasia within vermis of 13-year-old autistic male (**c**) with dysplastic granule neurons mixed with heterotopic (*arrow*) large cells (**d**) (B-5535). **e** Severe hypoplasia of cerebellar lobe 3 and unmodified lobe 6 (**f**), respectively, within the cerebellum of a 60-year-old autistic male (B-7090). In the affected region, the thickness of the hypoplastic molecular and granule cell layer was reduced by about 50%. Almost half of the dentate nucleus (*DN*) was less convoluted than the unaffected part (**g**)

that was diagnosed at the age of 14 months in a neuropathologically examined autistic male. The subependymal nodules detected in this autistic male's brain are partially similar to tubers seen in subjects diagnosed with tuberous sclerosis [24]. The cause of subependymal nodular dysplasia in the examined subject is unknown. In the reported subjects, bilateral periventricular nodules are linked to mutations of the filamin A (FLNA) gene located on chromosome Xp28. Filamin A is an actin-crosslinking protein that is essential for cell locomotion [16], and nodule formation might be related to a defect in cell migration. The presence of miniature nodules that were built of poorly differentiated small neurons within the subependymal cell layer and an increase in nodular size with signs of growth and differentiation of neurons suggests that neurogenesis, differentiation and maturation of neurons were in progress within the subependymal germinal matrix of the 7-year-old autistic child. This interpretation of subependymal nodule genesis is consistent with lineage studies demonstrating that cells in nodules express cellular markers that are typical for progenitors derived from the subventricular germinal zone [35, 67]. However, in contrast to the subependymal nodules seen in subjects with tuberous sclerosis, in the examined autistic subject, the nodules seen were small (from 258 to 3,310 µm in diameter), and did not have the characteristic ovoid or polygonal giant cells, 80–150 µm in diameter, giant cells with multiple and peripherally displaced nuclei [25], or balloon cells, which are considered the sine qua non histopathological features of the cortical tubers and subependymal nodules observed in tuberous sclerosis [73].

The enlarged caudate nucleus detected in the brain of the 7-year-old autistic subject is consistent with MRI reports documenting an increased volume of basal ganglia, including the caudate, in autism [54, 55, 66, 99]. A disproportionate increase of the caudate nucleus volume [66] suggests that in brains of some autistic individuals, extended neurogenesis within the subependymal cell layer may contribute to abnormal growth of the caudate nucleus. A similar process has been observed in the brains of people with Huntington disease, showing enhanced neurogenesis in the subependymal layer and suggesting renewal of the neuronal population in a degenerating caudate nucleus [26]. The caudate nucleus is a part of the fronto-striatal network involved in several functional domains that are impaired in autism, including lower order repetitive motor

behavior; intense circumscribed patterns of interests and higher order rituals and compulsions [41], and defects in cognitive functions [19, 109], planning and problem-solving skills [78, 98], short- and long-term memory [40] and learning [88].

Defective migration in autism

Heterotopia is a sign of altered migration leading to an abnormal distribution of gray matter nodular masses with disorganized or rudimentary lamination within the periventricular area (periventricular heterotopia) or subcortical white matter (subcortical heterotopia) [2]. In the examined cohorts, heterotopias were detected in the brains of four autistic subjects and in the brain of one control subject. Heterotopias are associated with mutations in the filamin 1 gene (FLNA1) [39, 46] and the chromosome X-linked DCX gene that codes for doublecortin, a protein expressed during brain development in migrating neurons, and in the cortical plate [29, 44, 45], which is involved in the formation of the microtubules necessary for neuronal migration [15]. Periventricular nodular heterotopia has been reported to be associated with pharmaco-resistant seizures in 80–90% of patients [31]. In the examined cohort, two periventricular heterotopias were detected in the brain of a child with subependymal nodular dysplasia and seizures diagnosed at 14 months of age (B-6403). Early onset epilepsy, diagnosed at the age of 4.5 months, might be related to the multiple heterotopias found within the frontal inferior gyrus, vermis and cerebellar white matter, coexisting with a focal cortical dysplasia and dentate gyrus dysplasia (B-5342).

Cortical, hippocampal and cerebellar dysplasia in autism

The most common form of developmental changes detected in the examined brains was focal dysplasia, which was observed in 11 (85%) of the autistic subjects. The morphology of focal dysplasias appears to reflect signs of abnormal migration, neuronal immaturity and altered cell arrangement, resulting in focal distortion of cytoarchitecture. In spite of similarities, the dysplastic changes in the neocortex and archicortex, dentate gyrus and cornu Ammonis and cerebellum also reveal a brain structure-specific pattern of dysplastic changes in autism.

Dysplasias encompass a spectrum of changes ranging from a mild form of cortical disruption, without cellular abnormalities, to the most severe form with cortical dyslamination, with abnormal morphology of neurons and astrocytes [93, 96, 107]. Focal cortical dysplasias with giant neurons and balloon cells [107, 113] are histopathologically similar to tubers containing giant cells in tuberous sclerosis complex [25, 73], suggesting a common pathogenic basis [113]. However, activation of the mammalian target of rapamycin (mTOR) pathway observed in the tuberous sclerosis complex is not present in focal cortical dysplasia [8, 80]. The giant neurons and ballooned cells, which are histopathological features of tuberous sclerosis and focal cortical dysplasia, were absent both in the subependymal nodules and in the focal cortical dysplasia observed in the examined autistic cohort. These findings suggest that in spite of similarities, the pathomechanisms of developmental alterations are different in the examined autistic subjects than those in tuberous sclerosis heterotopias or focal cortical dysplasia. The development of the giant neuron- and balloon cell-free dysplasias observed in the autistic subjects might be related to differences in cause and/or mechanism. The detection of changes similar to focal cortical dysplasia in association with prenatal ischemia [65] or in shaken infant syndrome [74] may support these speculations.

Ectopias and dysplastic changes were reported in the brains of autistic subjects, by several groups [4, 62–64, 91]. Bailey et al. [4] detected olivary dysplasia in the brain of three of the five autistic subjects, and ectopic neurons related to the olivary complex in two cases. Moreover, in the brains of four autistic subjects, cortical dysgenesis was found. In the brains of the autistic subjects, a strikingly consistent finding was cingulate cortex disordered lamination [62–64, 100]. A recent study of the cingulate cortex of nine autistic subjects revealed a developmental malformation with irregular lamination in three cases, and an increased number of neurons within the subcortical white matter in two [100]. Simms et al. [100] suggest that the excessive number of neurons in the subcortical white matter reflects the lack of proper resolution of the transient zone in the developing brain of autistic subjects. Studies by Fatemi et al. [37, 38] link the migration and lamination defects to a striking reduction of reelin (by 40%) and Bcl-2 (by 34–51%) in the brains of autistic subjects. Our studies along with others' suggest that in the majority of autistic subjects, heterotopias and dysplastic changes are the local sign of general developmental defects of migration with a broad spectrum of topographic, morphological, and functional outcomes.

In the examined brains of autistic subjects, signs of neuronal immaturity were a common finding. Failure of maturation of neuronal precursors caused by altered expression of cytoskeletal proteins and loss of neuronal polarity results in defects in migration to the destined layer and in incorrect vertical and horizontal orientation [93]. The immaturity of dysplastic neurons is reflected in the expression of a variety of proteins and mRNA that are not present in mature neurons an altered expression of developmentally regulated cytoskeletal elements [3, 23, 61, 76],

which are known to be crucial for dendrite arborization, spine formation, axon outgrowth and maintenance of cell size and shape. Reduced cell size, dendritic arborization and spine expression are characteristic of dysplastic neurons [6, 93]. Cortical dysplasias are the most epileptogenic lesions of the brain [107] and are observed in up to 25% of all epileptic surgeries [102]. More subtle cortical malformations or dysgenesis encountered in adults with epilepsy may lack the histological criteria for focal cortical dysplasia. They have been described as mild cortical dysplasia or microdysgenesis [77].

Microdysgenesis within the entorhinal cortex of the 23- and the 60-year-old autistic subjects in the examined cohort is unique because the selective deficit of neurons was limited almost exclusively to the stellate neurons in the second layer. It is possible that the observed dysgenesis is a result of defective migration of neurons to their intended destinations. The presence of a thicker molecular layer and the deeper location of islands in the entorhinal cortex of subjects with schizophrenia were previously interpreted as evidence that the stellate neurons do not reach their destinations during development, probably due to abnormal migration [36, 57]. Studies indicating the involvement of reelin and Bcl2 genes in the pathogenesis of schizophrenia [37, 47, 60] and the reduced expression of reelin and Bcl2 in people with autism suggest that these two genes play a role in abnormal brain development and contribute to the structural and functional anomalies seen in autism and schizophrenia [37].

The distortion of dentate gyrus development detected in two autistic subjects was reflected in granule cell migration into the molecular layer and formation of an additional granule cell layer. Distortion of the shape of the dentate granule cell layer with the formation of irregular circles and loops appears to be another piece of evidence suggesting abnormal neuronal migration and networking. Numerous factors up-regulate neurogenesis in the hippocampus [32], including seizures [70, 71], antidepressant drugs [59, 72] and lithium [18]. Several areas of dysplastic changes with significant deficits of pyramidal neurons were found in the CA1 sector in three autistic subjects, but thickening of the pyramidal layer and an increased packing of dysplastic neurons in the CA1 sector of the 56-year-old subject suggests a diversity of CA dysplasia patterns in autism. The lack of gliosis indicates that the observed pathology is a sign of microdysgenesis rather than an effect of hypoxic neuronal loss. A significant deficit of mature pyramidal neurons and the presence of small irregular or poorly differentiated oval neurons suggest the defect of neuronal maturation in autism.

We report a spectrum of focal developmental changes seen in the cerebellum of eight autistic subjects, including nodular (lobe X) [97] dysplasia in the cerebellum in five, vermal dysplasia in one, severe focal hypoplasia in one, and heterotopias in one other subject. The presence of heterotopias only in one control subject is evidence of a strong tendency for focal developmental changes of cerebellar microarchitecture that were present in 61% of the autistic subjects. Flocculonodular dysplasia affecting almost the entire lobe indicates that mechanisms leading to focal dysplasia, which were present in five (38%) of the autistic subjects, show extremely strong topographic predilection. The observed focal dysplasia was associated with profound local disorganization of granule cells, Purkinje cells and molecular layers limited to a small cerebellar compartment receiving major projections from the vestibular complex involved in the oculomotor and postural system. Similar cerebellar dysplastic changes classified as heterotaxias (clusters of poorly organized mixed cells) were identified in 14% of normal infants but in 83% of infants with trisomy of different chromosomes [92]. The presence within the dysplastic nodule of both GABAergic Purkinje cells produced from the cerebellar ventricular zone, and the glutamatergic granule neurons produced from the rhombic lip, and the preservation of the cytoarchitecture in the adjacent cerebellar folia suggest that the final steps of migration and networking are disturbed mainly or exclusively in the nodule of the majority of autistic subjects. The characteristic feature distinguishing lobule X from the other lobules is the abundance of the transcription factor Tbr2 positive unipolar brush cells (UBCs) [30, 34], which amplify inputs from vestibular ganglia and nuclei, by spreading and prolonging excitation within the internal granular layer [84]. Abnormal networking of Purkinje cells, granule neurons, and UBCs may contribute to altered cerebellar coordination of locomotion and motor learning and planning, as well as of higher cognitive processing [58]. Flocculonodular dysplasia appears to be another sign of the mosaic of local developmental defects, most likely predetermined by the spatial patterning of germinal zones in developing rhombic lip [110], and coexisting with more general developmental defects resulting in the accelerated growth of the brain in early childhood [89], minicolumn pathology [13, 14], reduced neuron volume [7, 108, 111], and desynchronized neuronal growth in many brain regions [111] observed in autism.

Identification of sub-groups with signs of hyperplasia, hypoplasia and normal-sized cerebellum [95] reflects the heterogeneity of the autistic population. Piven et al. [87] reported that cerebellar volume correlates with an increased total brain volume. In the majority of autistic subjects, reduced size of the cerebellar hemisphere is observed [42, 82], but this trend is not detectable in cohorts of high-functioning autistic individuals [56]. Regional hypoplasia affects the vermis in autistic individuals relatively often [20, 22, 52] and may be associated with the

deficits in attention-orienting [49, 104], stereotypic behavior and reduced exploration observed in autism [86]. In the examined autistic cohort, selective and severe hypoplasia of lobes 1–4 associated with hypoconvolution of a large portion of the dentate nucleus appears to correspond to clinically detected defects of movement coordination. These findings suggest that differences in the type, topography and severity of cerebellar developmental defects may contribute to different clinical manifestations.

In the 4–7-year-old autistic children examined in this study, the volume of the Purkinje cells was 38% smaller than that of the age-matched control group [111]. Moreover, it has been reported that Purkinje cells of the autistic subjects revealed a 40% decrease in the expression of glutamic acid decarboxylase 67 (GAD67) mRNA [114]. In autism, the basket cells provide an increased GABAergic feed-forward inhibition to Purkinje cells. The result could be disruption in the timing of Purkinje cell firings and altered inhibition of the cerebellar nuclei, which could directly affect cerebello-cortical output and contribute to the changes in motor behavior and cognition observed in autism [115]. These findings and the reduced volume (by 26%) of the neurons of the dentate nucleus seen in the 4–7-year-old autistic children [111] suggest that in autism, interactions between the Purkinje cells and dentate nucleus are modified on the structural, molecular and functional levels.

The (a) detected changes within the subependymal cell layer with subependymal nodular dysplasia, (b) subcortical and periventricular heterotopias and (c) neocortex, archicortex, dentate gyrus, cornu Ammonis and cerebellar dysplasia reflect focal modification of neurogenesis, migration and alterations of the cytoarchitecture of brain cortex, subcortical structures and cerebellum in autism. Detection of dysplastic changes only in one control brain and of the broad spectrum of focal developmental alterations in the brains of 92% of the autistic subjects indicates that focal changes are a reflection of global developmental abnormalities and that regional changes may have their own contribution to the clinical heterogeneity of autism.

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ORIGINAL ARTICLE

Differences Between the Pattern of Developmental Abnormalities in Autism Associated With Duplications 15q11.2-q13 and Idiopathic Autism

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Abstract

The purposes of this study were to identify differences in patterns of developmental abnormalities between the brains of individuals with autism of unknown etiology and those of individuals with duplications of chromosome 15q11.2-q13 (dup[15]) and autism and to identify alterations that may contribute to seizures and sudden death in the latter. Brains of 9 subjects with dup(15), 10 with idiopathic autism, and 7controls were examined. In the dup(15) cohort, 7 subjects (78%) had autism, 7 (78%) had seizures, and 6 (67%) had experienced sudden unexplained death. Subjects with dup(15) autism were microcephalic, with mean brain weights 300 g less (1,177 g) than those of subjects with idiopathic autism (1,477 g; $p < 0.001$). Heterotopias in the alveus, CA4, and dentate gyrus and dysplasia in the dentate gyrus were detected in 89% of dup(15) autism cases but in only 10% of idiopathic autism cases ($p < 0.001$). By contrast, cerebral cortex dysplasia was detected in 50% of subjects with idiopathic autism and in no dup(15) autism cases ($p < 0.04$). The different spectrum and higher prevalence of developmental neuropathologic findings in the dup(15) cohort than in cases with idiopathic autism may contribute to the high risk of early onset of seizures and sudden death.

Key Words: Autism, Chromosome 15q11.2-q13 duplication, Developmental brain alterations, Seizures, Sudden unexpected death.

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INTRODUCTION

Autism is the most severe form of autism spectrum disorder (ASD) and is characterized by qualitative impairments in reciprocal social interactions, qualitative impairments in verbal and nonverbal communication, restricted repetitive and stereotyped patterns of behavior, interests and activities, and onset before the age of 3 years (1). Autism is heterogeneous, both phenotypically and etiologically. In 44.6% of affected children, autism is associated with cognitive impairment as defined by intelligence quotient scores of less than 70 (2). Epilepsy is a comorbid complication diagnosed in up to 30% of individuals with autism (3). In 90% to 95% of cases, the etiology of autism is not known (idiopathic or nonsyndromic autism) (4, 5). Twin and family studies have indicated both a strong and a moderate heritability for ASD (6–8).

Approximately 5% to 10% of individuals with an ASD have an identifiable genetic etiology corresponding to a known single gene disorder, for example, fragile X syndrome or chromosomal rearrangements, including maternal duplication of 15q11-q13. Supernumerary isodicentric chromosome 15 [idic(15)] (formerly designated as inverted duplication 15)

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is a relatively common genetic anomaly that most often leads to tetrasomy or mixed trisomy/tetrasomy of the involved segments and arises from a U-type crossover between a series of low copy repeats (LCRs) located on the proximal long arm. Small heterochromatic idic(15) chromosomes, which do not include the 15q11-q13 region, are often familial and are not associated with a clinical abnormality (9, 10). The symptoms in people with idic(15) markers correlate with the extent of duplication of the Prader-Willi syndrome/Angelman syndrome critical region (15q11-q13) (10, 11). Larger supernumerary idic(15) chromosomes, which include the imprinted chromosome 15q11-q13 region, are associated with a cluster of clinical features that include intellectual deficits (IDs), seizures, autistic behavior, hypotonia, hyperactivity, and irritability (12). Duplications of chromosome 15q11-q13 account for approximately 0.5% to 3% of ASD and may be the most prevalent cytogenetic aberration associated with autism in most studies. These duplications range from 4 to 12 Mb and may occur either through generation of supernumerary idic(15) chromosomes or as interstitial duplications and triplications. For interstitial duplications, maternal origin confers a higher risk for an abnormal phenotype (13, 14), and most of the reported chromosome 15 duplications (dup[15]) are maternally derived. A small number of subjects with duplications of paternal origin have been variously reported as being unaffected (13, 15–17), affected but without ASD (16, 18), or affected with ASD (19). Interstitial triplications (int trp[15]) are relatively rare but have invariably been associated with a severe phenotype, including ID, ASD, or autistic features, and frequently with seizures. The parent-of-origin effect is not evident in the reported cases of int trp(15), with both maternal and paternal triplications associated with poor outcome.

Clinical studies indicate that most individuals diagnosed with dup(15) of maternal origin fulfill the criteria for the diagnosis of autism. In the first clinical reports, 24 individuals with idic(15) and autism were identified using standardized criteria of the *Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised* (13, 15, 20–24). In several other studies of subjects with idic(15) chromosomes, autism was clinically diagnosed, although without the application of standardized measures of autism (25–27). Application of the Gilliam Autism Rating Scale (28) to another idic(15) cohort confirmed a high prevalence of autism; 20 (69%) of 29 children and young adults with idic(15) had an ASD (29).

TABLE 1. Material Examined

Group	Case No.	Sex	Age, y	Cause of Death	PMI, h	Hem	Brain Weight, g
dup(15) autism	1	M	9	SUDEP	13.6	R	1,130
	2	M	10	SUDEP	17.7	R	1,070
	3	M	11	SUDEP	10.5	R	1,540
	4	F	15	SUDEP (suspected)	24.0	LR	1,141
	5	F	15	Pneumonia	—	LR	1,125
	6	M	20	Cardiopulmonary arrest (choking on food)	28.1	L	1,190
	7	M	24	Pneumonia	36.3	L	1,200
	8	F	26	SUDEP (suspected)	28.6	R	1,310
	9	F	39	SUDEP	32.8	R	890
			Mean (SD)		23.9 (9.2)		1,177 (177)
Idiopathic autism	1	M	2	Asphyxia (drowning)	4.0	R	1,328
	2	F	5	Asphyxia (drowning)	33.0	R	1,360
	3	M	5	Asphyxia (drowning)	25.5	R	1,560
	4	M	8	Asthma attack	13.8	R	1,740
	5	M	9	SUDC	3.7	R	1,690
	6	M	11	Asphyxia (drowning)	—	R	1,400
	7	M	28	Seizure-related	43.0	R	1,580
	8	M	32	Brain tumor (glioblastoma multiforme)	—	L	1,260
	9	M	51	Heart failure	22.2	R	1,530
	10	M	52	Heart failure	11.5	L	1,324
			Mean (SD)		19.6 (14.0)		1,477 (166)
Control	1	F	8	Rejection of cardiac transplant	20.0	R	1,340
	2	M	14	Asphyxia (hanging)	5.0	R	1,420
	3	M	14	Multiple traumatic injuries	16.0	R	1,340
	4	M	32	Heart failure	14.0	R	1,401
	5	F	33	Bronchopneumonia	6.0	L	1,260
	6	F	43	Sepsis	10.1	L	1,350
	7	M	47	Myocardial infarct	23.0	L	1,450
			Mean (SD)		13.4 (6.8)		1,366 (63)

PMI, postmortem interval; Hem, hemisphere; R, right; L, left; SUDEP, sudden unexpected death of subject with known epilepsy: SUDC, sudden unexpected death in childhood.

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TABLE 2. Chromosome 15 Abnormalities in the dup(15) Cohort

Case #	Chromosomal Alterations	Prader-Willi Syndrome/Angelman Syndrome Critical Region (PWS/ASCR)	Parental Origin of Abnormality
1	47,XY,+idic(15)(q13;q13); Idic(15) arising from BP3:BP3 exchange. Subject 02-18, Wang et al (47)	Tetrasomy	Maternal
2	47,XY,+idic(15)(q13;q13); Idic(15) arising from BP4:BP5 exchange. Subject 01-22, Wang et al (47)	Tetrasomy	Maternal
3	47,XY,+der(15)(pter>q13::q13>cen>q13::q13>pter). Tricentric chromosome 15 arising from BP3:BP3 exchanges. Case 2, Mann et al (46); Subject 00-29, Wang et al (47)	Hexasomy	Maternal
4	47,XX,+idic(15)(q13;q13); Idic(15) arising from BP4:BP5 exchange. Subject 99-93, Wang et al (47)	Tetrasomy	Maternal
5	47,XX,+der(15)(q13;q13); Idic(15) arising from BP4:BP5 exchange	Tetrasomy	Maternal
6	IDIC15 (2 extra copies of region from beginning of array to BP4)	Tetrasomy	Not determined
7	47,XY,del(15)(q11.2)+idic(15)(q13;q13); Idic(15) arising from BP4:BP5 exchange; deletion of BP1:BP2 on 1 homolog of chromosome 15. Subject 00-03, Wang et al (47)	Tetrasomy	Maternal
8	47,XX,+idic(15)(q13;q13); Idic(15) arising from BP4:BP5 exchange. Subject 99-27, Wang et al (47)	Tetrasomy	Maternal
9	46,XX,trp(15)(q11.2q13). Subject 02-9, Wang et al (47)	Tetrasomy	Maternal

PWS/ASCR, Prader-Willi syndrome/Angelman syndrome critical region.

The link between the extent of genetic duplication and clinical phenotype has not yet been determined, but genomic and functional profiling provides insights into the direct and indirect effects of the copy number gains associated with chromosome 15 duplications. The γ-aminobutyric acid type A ($GABA_A$) receptor subunit genes ($\alpha 5$, β, and $\gamma 3$) that have been implicated in the etiology of autism (30) are located in the susceptibility segment of duplicated chromosome 15

TABLE 3. Autism Diagnostic Interview-Revised–Based Diagnosis of Autism

Group	Case No.	Reciprocal Social Interactions (10)	Communication: Verbal (8)	Communication: Nonverbal (7)	Restricted, Repetitive, and Stereotyped Behavior (3)	Alterations Evident Before 36 mo (1)	Diagnosis (Test)
dup(15) autism	1	7	NA	0	0	5	Autism (ADOS-G)
	2	24	NA	11	5	3	Autism (ADI-R)
	3	18	NA	2	2	5	PDD-NOS
	4	26	22	NA	12	5	Autism (ADI-R)
	5	—	—	—	—	—	Autism (ADI-R) (score not available)
	6	27	18	NA	4	5	Autism (ADI-R)
	7	23	22	NA	9	3	Autism (ADI-R)
	8	28	16	NA	9	5	Autism (ADI-R)
	9						Unknown
Idiopathic autism	1	14	NA	9	6	5	Autism (ADI-R)
	2						Autism (ADOS)
	3	22	NA	14	6	5	Autism (ADI-R)
	4	11	NA	8	2	4	Atypical autism – ASD (ADI-R)
	5	26	NA	12	5	4	Autism (ADI-R)
	6	25	20	NA	4	4	Autism (ADI-R)
	7	22	16	NA	5	3	Autism (ADI-R)
	8	—	—	—	—	—	Autism (ADI-R) (score not available)
	9	27	19	9	6	5	Autism (ADI-R)
	10	—	—	—	—	—	Atypical autism – ASD (ADI-R score not available)

ADI-R, Autism Diagnostic Interview – Revised (cutoff scores); ADOS-G, Autism Diagnostic Observation Scale – Generic (42); NA, not applicable; PDD-NOS, Pervasive Developmental Disorder – Not Otherwise Specified.

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TABLE 4. Behavioral, Neurologic, and Other Clinical Observations

Group	Case No.	Psychiatric Disorders and Neurologic Symptoms	Cognitive Assessment	Seizures (Age at Onset)
dup(15) autism	1	Severe hypotonia. Regression in infancy. Abnormal response to pain and heat.	Profound ID (DQ < 20)	Infantile spasms. Intractable epilepsy (10 mo)
	2	Hypotonia. Severe regression at age of 15 mo. Head banging.	Profound ID (DQ = 22)	Intractable epilepsy (8 mo)
	3	Regression with infantile spasms. Severe hypotonia.	Profound ID (DQ < 20)	Infantile spasms. Intractable epilepsy (10 mo). Vagus nerve stimulator.
	4	Delay of motor skills. Mild to moderate spastic quadriparesis. Abnormal response to pain, cold.	Severe ID (DQ = 31)	Seizures (11 y)
	5	Hyperactive, verbal	(—)	No record
	6	Sleep disorder. Abnormal response to pain, heat and cold.	(—)	No record
	7	Abnormal gait	Profound ID (DQ < 20)	Intractable epilepsy (7 y). Vagus nerve stimulator. Callosotomy.
	8	Obsessive compulsive symptoms	Moderate ID (IQ = 36)	Epilepsy (16 y)
	9	Cerebral palsy. Microcephaly	Severe ID.	Intractable epilepsy (9 y). Vagus nerve stimulator.
		Aggressive behavior. Trichotillomania.	(—)	
Idiopathic autism	1	Self-stimulatory behavior	(—)	No record
	2	Hyperactivity, attention deficit. Sleep disorder. Enhanced sensitivity to light and sound.	Cognitive delay (IQ = 65)	No record
	3	Sleep disorder	(—)	No record
	4	Self-stimulatory behavior	(—)	Epilepsy (8 y)
	5	Hypotonia	Moderate ID	No record
	6	No record	Moderate ID	Epilepsy
	7	Bipolar disorder	(—)	Epilepsy
	8	No record	(—)	No record
	9	Enhanced sensitivity to sound, heat, and cold. Low pain threshold.	(—)	One grand malseizure
	10	Bipolar disorder, social anxiety	(—)	No record

DQ, developmental quotients; ID, intellectual deficit; —, no formal assessment of ID available.

(31–34). A map of parent-of-origin-specific epigenetic modifications suggests that this imprinted locus may have links not only with autism but also with other psychiatric phenotypes (35). Differential methylations in the 15q11-q13 region, including the $GABA_A$ gene (30, 36–38), may contribute to epigenetic modifications and a broader clinical phenotypes in dup(15)/autism. Several other genes located in or near the 15q11-q13 region may contribute to a variable phenotype of autism, including a gene for juvenile epilepsy located near D15S165 (39) and a locus for agenesis of the corpus callosum (40).

We hypothesized that the neuropathology of autism with dup(15) differs from that of idiopathic autism and that it would provide an explanation for the high prevalence of seizures and associated sudden death in the dup(15) cohort. The aim of this comparative postmortem study of the brains of individuals diagnosed with idic(15) or int trp(15) (collectively referred to as dup[15]) and of individuals with idiopathic autism was to identify common neuropathologic developmental defects for both cohorts and the patterns of changes distinguishing dup(15) from idiopathic autism. The dup(15) cohort examined exhibited a strikingly high prevalence of epilepsy, including intractable epilepsy, and a high rate of sudden unexpected death in childhood and early adulthood. Therefore, the second aim of the study was to identify patterns of neuropathologic changes that may contribute to epilepsy and sudden death in the dup(15) cohort.

MATERIALS AND METHODS

The cohort of subjects diagnosed with dup(15) consisted of 9 subjects (range, 9–39 years), including 5 males (55%) and 4 females (45%). The cohort with autism consisted of 10 subjects (range, 2–52 years), including 9 males (90%) and 1 female (10%). The control cohort consisted of 7 subjects from 8 to 47 years, including 4 males (43%) and 3 females (57%). The brain of 1 subject diagnosed with dup(15) was excluded because of very severe autolytic changes, and the brain of 1 control subject was excluded because of lack of information about cause of death. The mean postmortem interval varied from 23.9 hours in the dup(15) cohort, to 19.6 hours in the idiopathic autism cohort, and 13.4 hours in the control group (Table 1).

Clinical and Genetic Characteristics

Psychological, behavioral, neurologic, and psychiatric evaluation reports and reports by medical examiners and pathologists were the source of the medical records of the examined postmortem subjects. Medical records were obtained after consent for release of information from the subjects' parents. In

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TABLE 5. Topography and Type of Major Developmental Alterations

		Hippocampus		Cerebral cortex	Cerebellum	
Group	**Case No.**	**Heterotopia (Alveus, CA4, DG)**	**Dysplasia (DG)**	**Dysplasia**	**Heterotopia**	**Dysplasia**
dup(15) autism	1	+	++		+	
	2	+	++			+
	3	+	++		+	+
	4	+				+
	5	+	+++		++	
	6	+	++			+
	7	+	+++++		++	+
	8		+++		+	+
	9	++	+++			—
	8 (89%)	8 (89%)	0	5 (56%)	6/8 (75%)	
Idiopathic autism	1		+	+	+	+
	2			+		+
	3			+		+
	4	+		+	+	+
	5				+	
	6				+	—
	7			+	+	
	8				+	
	9					—
	10					
	1 (10%)	1 (10%)	5 (50%)	6 (60%)	4/8 (50%)	
Control	1					
	2					
	3					
	4					
	5					
	6					
	7					+
		0	0	0	0	1 (14%)
p						
dup(15) vs idiopathic autism		0.001	0.001	0.03	ns	ns
dup(15) vs control		0.001	0.001	ns	0.03	0.04
Autism vs control		ns	ns	0.04	0.03	ns

+, The number of types of developmental alterations and percentages of subjects with developmental defects are in parentheses; ns, not significant; —, missing structure; DG, dentate gyrus.

Statistical analyses: Fisher exact test and Mann-Whitney *U* test.

most subjects diagnosed as being affected by dup(15) and idiopathic autism, the Autism Diagnostic Interview – Revised (ADI-R) was administered to the donor family after the subject's death as a standardized assessment tool to confirm an autism diagnosis (41). In addition, 6 of the subjects with dup(15) chromosomes were enrolled in a study of molecular contributors to the phenotype. The study was approved by the institutional review boards of the University of California, Los Angeles, and Nemours Biomedical Research. Before their deaths, 6 subjects (Cases 1–4, 7, and 8) had undergone behavioral and cognitive testing using the ADI-R, the Autism Diagnostic Observation Scale – Generic (ADOS-G) (42), and the Mullen Scales of Early Learning (43, 44) or the Stanford-Binet Intelligence Scales (45). Age at evaluation ranged from 45 to 251 months.

Molecular genetic evaluation using antemortem peripheral blood samples and lymphoblast cell lines for 8 of the dup(15) cases included genotyping with 19 to 33 short tandem repeat polymorphisms from chromosome 15, Southern blot analysis of dosage with 5 to 12 probes, and measurement of the methylation state at *SNRPN* exon α, as described (46). In addition, array comparative genomic hybridization was performed, using a custom bacterial artificial chromosome array (47). Morphology of the duplication was confirmed by fluorescent in situ hybridization using 5 to 8 probes that detect sequences on chromosome 15q11-q14 (46).

Tissue Preservation for Neuropathologic Study

One brain hemisphere from each subject was fixed in 10% buffered formalin, dehydrated in a graded series of ethanol, infiltrated with polyethylene glycol 400 (no. 807 485; Merck, Whitehouse Station, NJ) and embedded in fresh polyethylene glycol 1000 (48) and stored at 4°C. Tissue blocks were then cut at a temperature of 18°C into 50-μm-thick

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serial sections identified with a number and stored in 70% ethyl alcohol at room temperature. Free-floating hemispheric sections were stained with cresyl violet and mounted with Acrytol. To increase the probability of detection of small focal developmental defects, on average, 140 hemispheric cresyl violet–stained sections at a distance of 1.2 mm were examined per case.

Tissue acquisition for this project was based on individual tissue transfer agreements between the project's principal investigator and several tissue banks, including (i) the National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders at the University of Maryland School of Medicine, Baltimore, MD; (ii) the Harvard Brain Tissue Resource Center, McLean Hospital, Belmont, MA; and (iii) the Brain and Tissue Bank for Developmental Disabilities and Aging of the New York State Institute for Basic Research in Developmental Disabilities. The institutional review board of the Institute for Basic Research in Developmental Disabilities approved the methods applied in this study.

Statistical Analysis

Differences among groups in the presence or absence of heterotopias and dysplasias were examined using the Fisher exact test. Differences in the numbers of abnormalities were examined using the Mann-Whitney *U* (Wilcoxon signed ranks) test or (for comparisons of all 3 groups) the Kruskal-Wallis one-way analysis of variance (an extension of the *U* test), with exact probabilities computed using version 12 of the SPSS statistical package (SPSS for Windows, Release 12.0, 2003; SPSS, Inc, Chicago, IL). Brain weights were compared in one-way analysis of variances controlled for age.

RESULTS

Genetic Characteristics

For 8 subjects (Cases 1–5 and 7–9) in the dup(15) cohort, duplication chromosomes was characterized using a combination of genotyping, fluorescent in situ hybridization, Southern blot, and array comparative genomic hybridization with lymphoblasts generated from antemortem blood samples (Table 2). All were maternally derived; 7 of these subjects were tetrasomic for the imprinted region between brake points (BP)2 and BP3, although the BP involved was variable. The idic(15) present in cells from Case 1 was generated by an exchange between copies of LCR3, causing tetrasomy that extended only to BP3. Four subjects (Cases 2, 4, 5, and 8) had the most common form of idic(15) chromosomes arising by nonallelic homologous recombination (NAHR) between BP4 and BP5, leading to tetrasomy of the region between the p-arm and BP4, with trisomy for the interval between BP4 and BP5. Another subject (Case 7) had a similar idic(15) chromosome but also carried a deletion between BP1 and BP2 on 1 homolog of chromosome 15 (Subject 00-03) (47). Case 3 had a complex tricentric supernumerary chromosome arising from NAHR between multiple copies of BP3, rendering him hexasomic for the region between the centromere and BP3 (46). One subject (Case 9) had an int trp(15) chromosome that led to tetrasomy between BP1 and BP4 and trisomy for the interval between the fourth and fifth LCRs, similar to the dosage seen in the BP4:BP5 idic(15) chromosomes.

The study of 5 subjects with idiopathic autism (Cases 1–4 and 9) revealed the absence of the relevant 15q11-q13 deletion or duplication between BP2 and BP3. In CAL105 normal karyotype was found. In 4 subjects (Cases 5, 7, 8, and 10), frozen tissue and genetic data were not available.

Clinical Characteristics

Of the 9 subjects with dup(15), 7 (78%) were diagnosed with autism (Table 3). In 6 cases, autism was diagnosed clinically and was confirmed with postmortem ADI-R. In a 9-year-old boy (Case 1), autism was diagnosed with ADOS-G (Table 3). This case was reported as Case 2 in Mann et al (46). An 11-year-old boy (Case 3) revealed impairments consistent with the diagnosis of pervasive developmental disorder – not otherwise specified.

In the idiopathic autism group, all 10 subjects were diagnosed clinically as having an ASD. Postmortem ADI-R confirmed a classification of autistic disorder in 6 cases. Two subjects, an 8-year-old boy (Case 4) and a 52-year-old man (Case 10), were diagnosed with atypical autism or high-functioning autism. In a 5-year-old girl (Case 2), autism was diagnosed with ADOS-G. A 32-year-old man (Case 8) and a 52-year-old man (Case 10) were clinically diagnosed as having autism, but the ADI-R could not be conducted postmortem owing to the unavailability of a caregiver who could report on their behavior as a child.

Among the 9 examined subjects with dup(15) chromosomes, 7 individuals were diagnosed with seizures (78%) (Table 4). In 6 cases, death was sudden and unexplained in patients with epilepsy (SUDEP, 6/9 [67%]). In the 10 subjects with autism, epilepsy was diagnosed in 3 (30%) and death was seizure related in 1 (10%; Table 1). In a previously described cohort of 13 subjects with autism who were subject to postmortem examination (49), seizures were reported in 6 (46%) and death was seizure related in 4 (31%).

Brain Weight

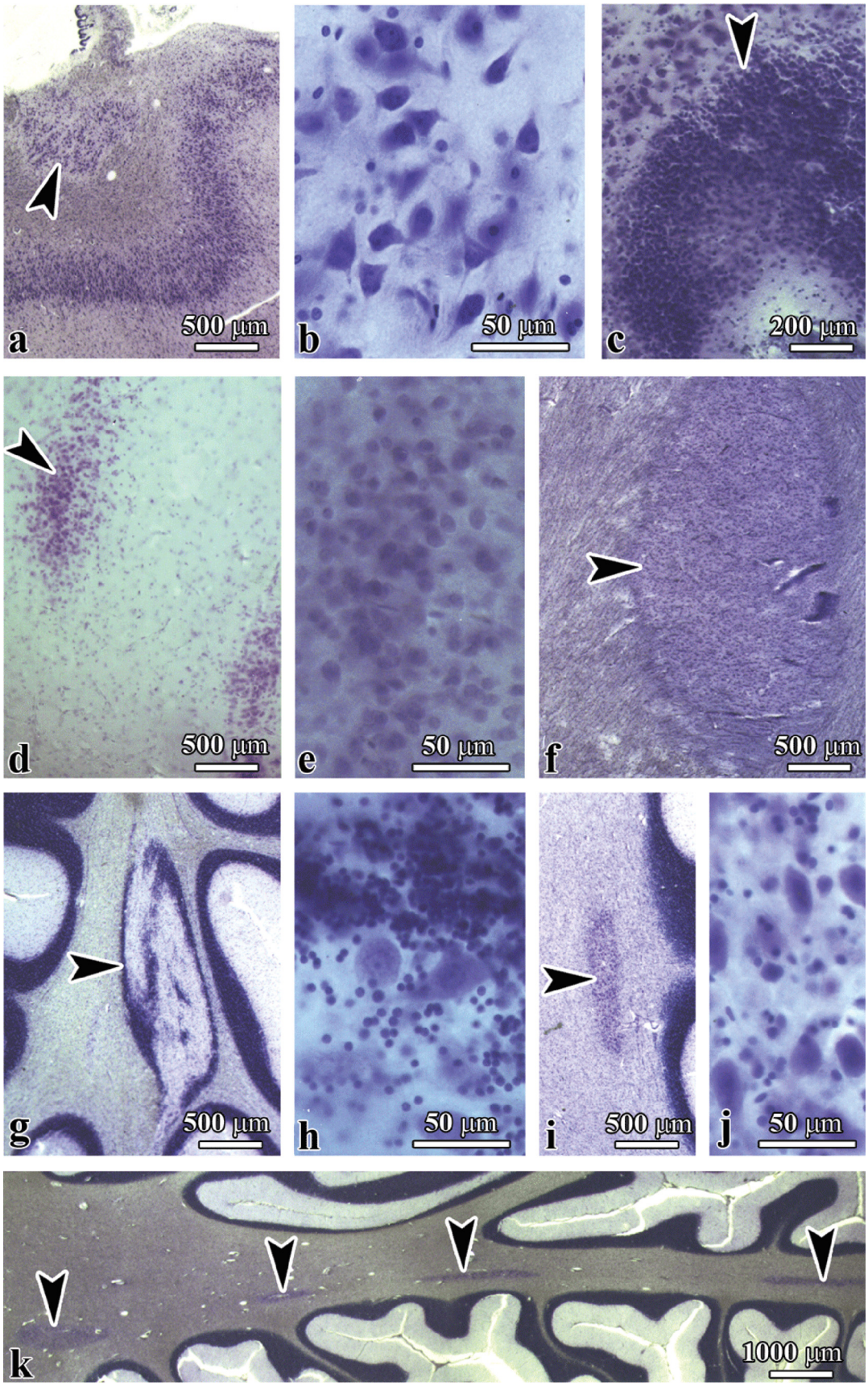
The mean brain weight in dup(15) autism was 1,177 g, 300 g less than in idiopathic autism and 189 g less than in the control group (Table 1). Age-adjusted means for these 3 groups were 1,171, 1,474, and 1,378 g, respectively ($F_2 = 9.79$, $p < 0.001$). Post hoc tests showed the difference between the idiopathic and dup(15) groups with autism to be significant (Scheffé-corrected $p = 0.001$). The difference between the dup(15) and control groups was not significant, although suggestive ($p = 0.06$). The difference between the idiopathic and control groups was not significant.

Developmental Abnormalities in Autism Associated With dup(15) and Idiopathic Autism

Three major types of developmental changes, including (i) heterotopias, (ii) dysplastic changes, and (iii) defects of proliferation resulting in subependymal nodular dysplasia, were detected in both the dup(15) (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A323) and idiopathic autism (Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A324) cohorts. In the control group,

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1 subject had small cerebellar subependymal heterotopias and 2 cases had dysplastic changes in the cerebellar flocculus and nodulus (Table, Supplemental Digital Content 3, http://links.lww.com/NEN/A325). Although all 9 dup(15) subjects and all 10 subjects with autism had developmental abnormalities, there were significant differences between the dup(15) autism and idiopathic autism cohorts in the number of developmental defects and their distribution (Table 5).

Heterotopias

Migration developmental abnormalities were detected as heterotopias in the hippocampal alveus, the CA4 sector, the dentate gyrus (DG) molecular layer, and the cerebral and cerebellar white matter (Fig. 1). A description of each is given below.

Heterotopias in the Hippocampus

A relatively large proportion of heterotopic cells in the alveus had the morphology of pyramidal neurons, although they were much smaller than neurons in the cornu Ammonis and were spatially disoriented. Heterotopias composed of neurons with the morphology of granule neurons of the DG were detected in the CA4 sector and in the molecular layer of the DG. Heterotopias in the alveus, CA4, and DG were found in 8 dup(15) subjects (89%), 1 subject with idiopathic autism (10%), and no control subjects. This difference between dup(15) autism and idiopathic autism cohorts ($p < 0.001$) and dup(15) and control subjects was highly significant ($p < 0.001$; Table 5). The difference between the idiopathic autism and control groups was not significant.

Heterotopias in the Cerebellar White Matter

The morphology of the cerebellar heterotopias reflected 2 types of migration defects. The presence of a mixture of granule and Purkinje cells suggests that clusters of cerebellar cortical neurons do not reach their destination site (Type 1). The second type of cerebellar heterotopia (Type 2) is composed of 1 type of cells with the morphology of cerebellar deep nuclei neurons. Both types of heterotopias were composed of neuronal nuclear marker–positive cells mixed with glial fibrillary acidic protein–positive astrocytes (not shown). In some cases, multiple Type 1 and 2 heterotopias were detected in the cerebellar white matter. In contrast to the significant difference in the prevalence of hippocampal heterotopias, there was no significant difference in the prevalence of heterotopias in the cerebellar white matter between the dup(15) (56%) and idiopathic autism (60%) groups (Table 5). However, the differences between the dup(15) autism and control groups ($p < 0.04$) and between the idiopathic autism and control groups ($p < 0.04$) were significant. Heterotopias in cerebral white matter were rare in both the dup(15) (1/9; 11%) and idiopathic autism (1/10; 10%) groups (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A323, and Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A324).

Dysplasias

Dysplastic changes reflect focal microdysgenesis in the hippocampal DG and cornu Ammonis, the amygdala, and the cerebral and cerebellar cortices. Several types of dysplasia were detected in the DG, including hyperconvolution of the DG, duplication of the granular layer distorting the architecture of the molecular layer of the DG, irregular protrusions of the granular layer into the molecular layer, focal thinning and/or thickening of the granular layer, and fragmentation of the granular layer with the formation of isolated nests of granule cells (Fig. 2). The susceptibility of the DG to developmental abnormalities was several times more apparent in dup(15) syndrome than in idiopathic autism cases. They were detected in 8 (89%) of 9 subjects in the dup(15) group and in only 1 subject with autism (1/10, 10%; $p < 0.001$; Table 5). The number of different types of developmental abnormalities in the dup(15) group ranged from 2 per case in 4 subjects, to 3 per case in 3 subjects, and 5 types in 1 case. The total number of different types of dysplasia was 22 times greater in the dup(15) cohort than in the idiopathic autism cohort ($p < 0.001$). The difference between idiopathic autism (1 positive case) and the control group (no dysplastic changes in the DG) was not significant.

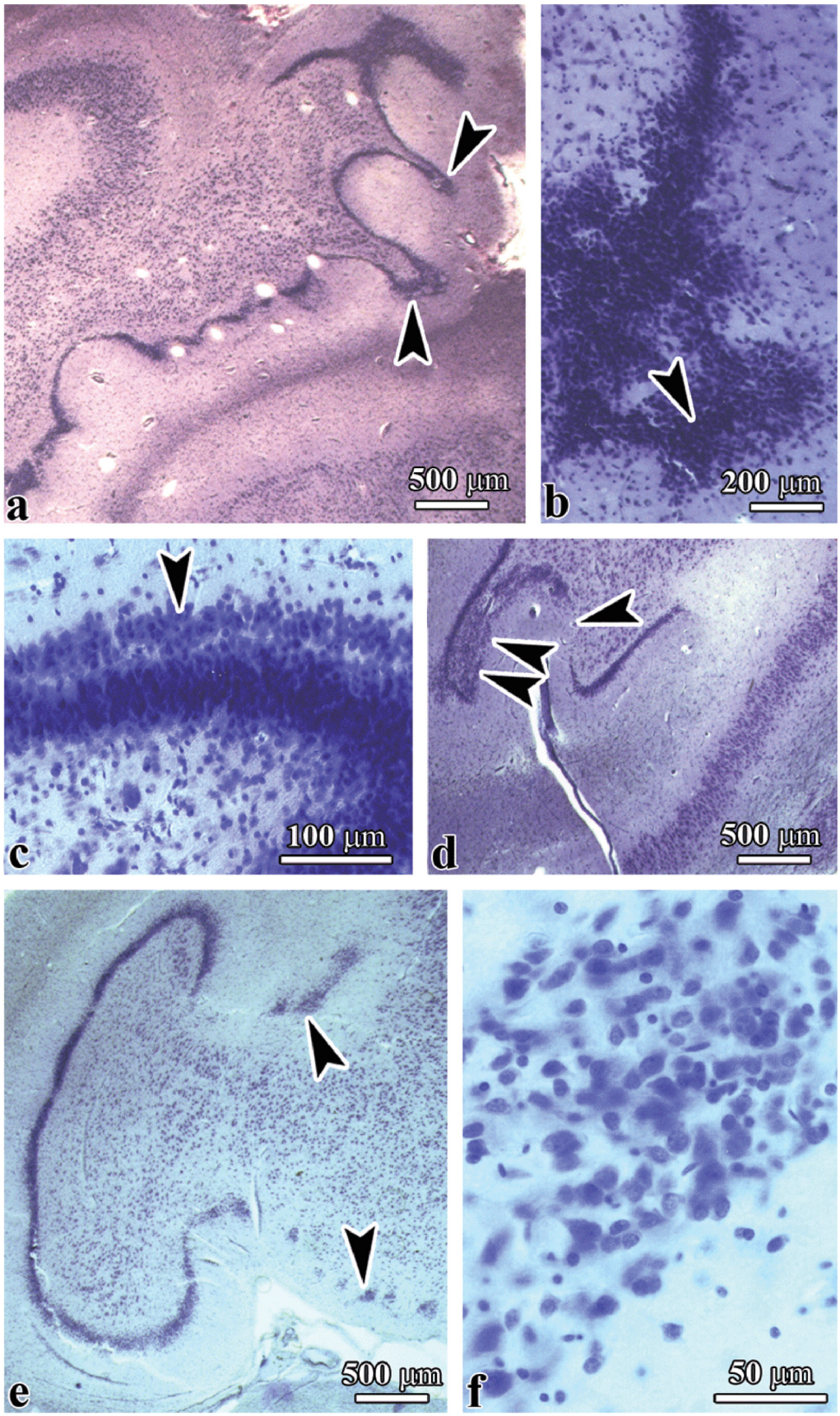
The spectrum of dysplastic changes in the cornu Ammonis comprised abnormal convolution of the CA1 sector, focal deficits of pyramidal neurons, and distortion of the shape, size, and spatial orientation of pyramidal neurons, clustering of dysplastic neurons in the CA1 sector, and many foci of severe microdysgenesis in the CA4 sector, with clustering of immature neurons (Figs. 3A–E). Dysplastic changes in the amygdala resulted in multiple irregular nests of 20 to 40 cells composed of relatively few small immature neurons and numerous oval or bipolar hyperchromatic neurons that were larger than normal amygdala neurons (Fig. 3F). Dysplastic changes in the cornu Ammonis were detected in 2 subjects with dup(15) syndrome and in 2 brains of subjects with autism (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A323, and Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A324).

The presence of dysplastic changes in the cerebral cortex of 5 of the 10 subjects with idiopathic autism (50%) was in striking contrast to the absence of these changes in the dup(15) ($p < 0.03$) and control subjects ($p < 0.04$; Table 5). Three types of cerebral cortex dysplasia were found in the

FIGURE 1. Topography and morphology of heterotopias in the brains of individuals with duplications of chromosome 15q11.2-q13 (subjects with dup[15] and autism). **(A, B)** Heterotopia (**A**, arrowhead) in the alveus composed of bipolar, multipolar, and pyramidal-like neurons shown at higher magnification in **B**. **(C–E)** Heterotopias composed of cells with the morphology of granule neurons in the CA4 sector (**C,** arrowhead) and in the molecular layer (arrowhead) of the dentate gyrus **(D)**; higher magnification is shown in **E**. **(F)** Heterotopia (arrowhead) in the cerebral white matter. **(G–J)** Heterotopias in the cerebellum consist of mixed components of the cerebellar cortex (**G**, arrowhead), including granule and Purkinje cells **(H)**; one is composed of one type of neuron (**I**, arrowhead), with the morphology of cells of cerebellar deep nuclei **(J)**. **(K)** Multifocal heterotopias of both types in cerebellar white matter (arrowheads). **(A–C, F)** dup(15), Case 2; **(D, E)** dup(15), Case 6; **(G–J)** dup(15), Case 5; **(K)** idiopathic autism, Case 5.

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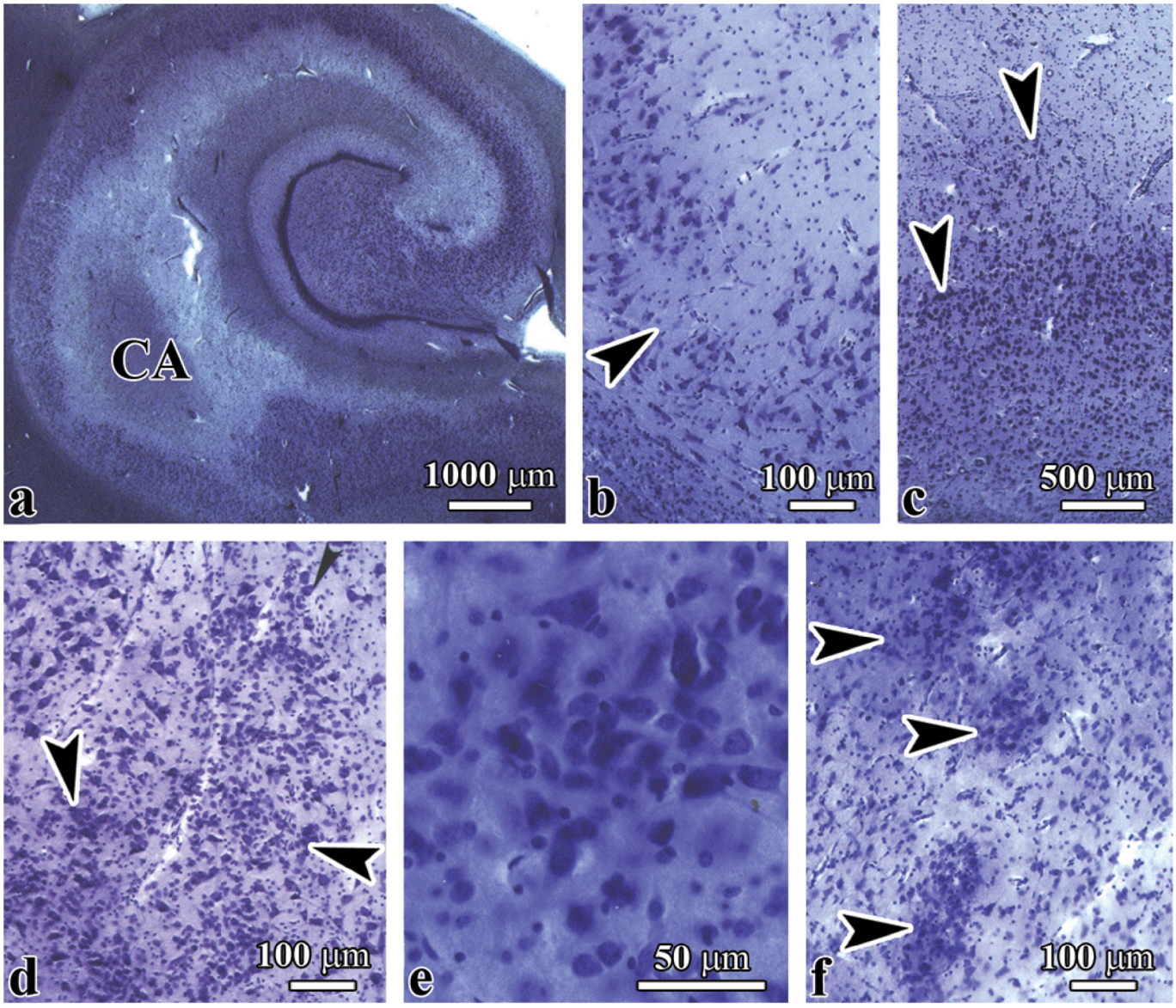


FIGURE 3. Multiple dysplastic changes in the cornu Ammonis (CA) and amygdala in an 11-year-old boy with hexasomy of chromosome 15q11.2q13 (Case 3). **(A–C)** There is abnormal convolution of the CA1 sector **(A)** with focal microdysgenesis of the pyramidal layer **(B**, arrowhead**)** and clustering of disoriented polymorphic neurons **(C**, arrowheads**)**. **(D, E)** Marked multifocal microdysgenesis in the CA4 sector **(D**, arrowheads**)**, with clustering of a mixture of small and large polymorphic neurons **(E)**. **(F)** Multifocal microdysgenesis (arrowheads) in the amygdala is composed of small immature neurons and neurons that are larger than normal amygdala neurons.

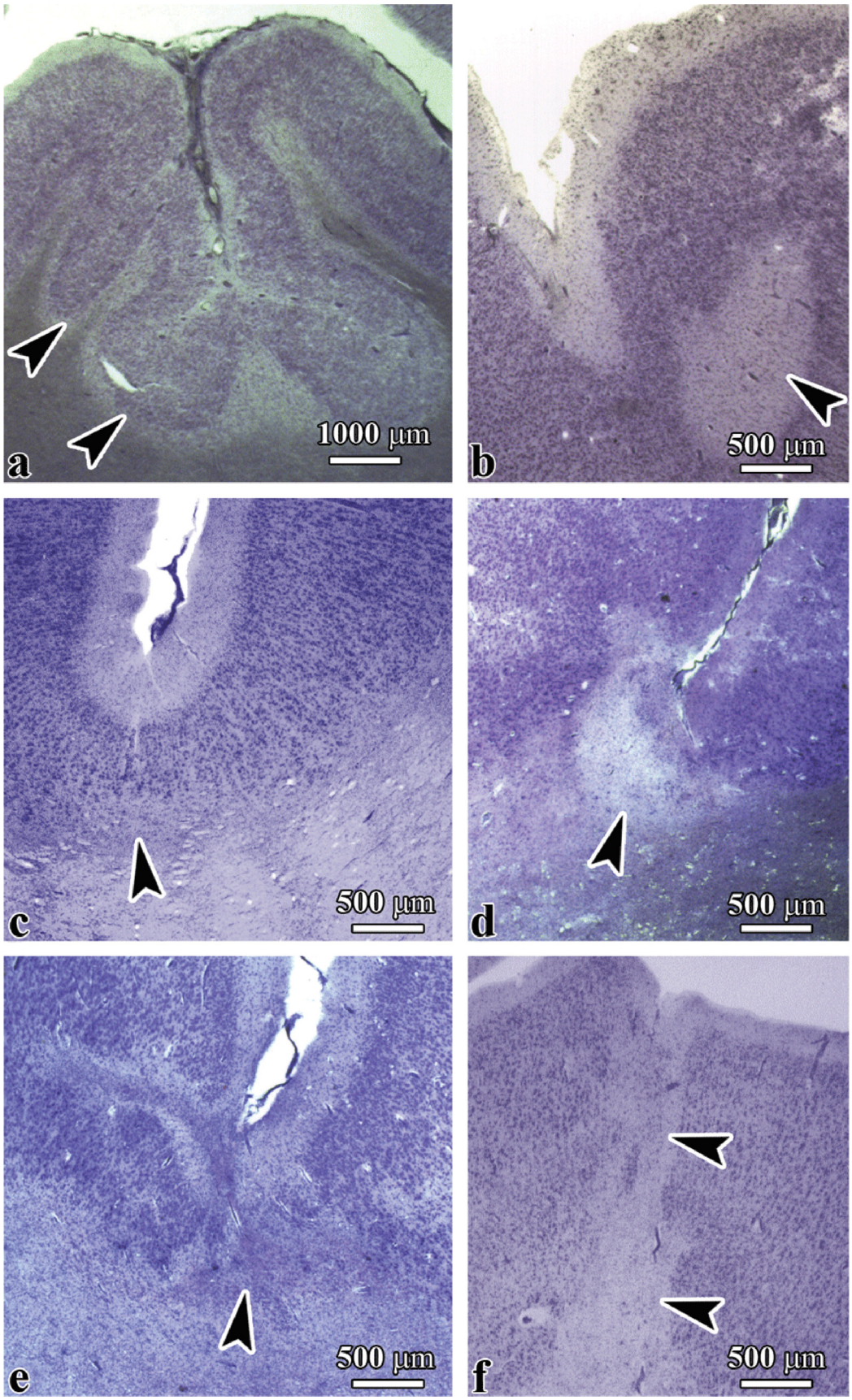
idiopathic autism group: focal polymicrogyria, multifocal cortical dysplasia, and bottom-of-a-sulcus dysplasia (Supplemental Digital Content 2, http://links.lww.com/NEN/A324).

Focal polymicrogyria, which reflects a gyrification defect, was found in the frontal lobe in an 8-year-old boy with autism diagnosed with ASD (Case 4), which resulted in local abnormal folding of the cortex with formation of numerous small and irregular microgyri and distortion of the cortical thickness and vertical/horizontal cytoarchitecture (Fig. 4A). The most common developmental abnormality was cortical

FIGURE 2. Six types of dysplastic changes in the dentate gyrus of subjects diagnosed with duplications of chromosome 15q11.2-q13 (dup(15]) syndrome. **(A–F)** Hyperconvolution of the dentate gyrus within the hippocampal body **(A)**, irregular large protrusions of the granular layer **(B)**, duplication of the granular layer **(C)**, focal thinning and discontinuity of granular layer (arrowhead), and thickening of the granular layer confirmed by examination of serial sections (**D**, 2 arrowheads), hippocampal malrotation and granular layer fragmentation into small clusters of cells of irregular shape and variable size **(E, F)**. **(A)** dup(15), Case 8; **(B)** dup(15), Case 7; **(C, E, F)** dup(15), Case 3.

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dysplasia with focal hypocellularity or acellularity and loss of cortical vertical and horizontal cytoarchitecture (Fig. 4B).

Another observed gyrification defect was multifocal bottom-of a-sulcus dysplasia with selective changes in the deepest layer, expansion of dysplastic changes to 2 to 3 deep layers of the cortex, or affecting the entire thickness of the cortex. This developmental abnormality was most often seen in the superior frontal and temporal gyrus, the Heschl gyrus, the middle temporal gyrus, the insula, and the parahippocampal gyrus in a 5-year-old girl diagnosed with idiopathic autism (Case 2; Figs. 4C–F).

Three types of dysplastic changes were found in the cerebellum of the dup(15) subjects and the subjects with autism. These included dysplasia in parts of the nodulus and flocculus, vermis dysplasia, and focal polymicrogyria (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A323, and Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A324). In the nodulus and flocculus, dysplasia resulted in total spatial disorganization of the granular, molecular, and Purkinje cell layer; only a few small abnormally branched Purkinje cells were found to be dispersed among the granule cells in the affected areas. There were many interindividual differences observed in the nodulus or flocculus volume affected by dysplastic changes. Cerebellar dysplasia was commonly observed in both cohorts. Nodulus dysplasia was present in 7 (87%) of 8 dup(15) subjects and in 6 (75%) of 8 subjects with autism (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A323, and Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A324). Flocculus dysplasia was detected in 6 (75%) of 8 dup(15) subjects, in 4 (50%) of 8 subjects with autism, and in 1 (14%) of 7 control subjects (Table 5). The difference between the groups with autism was not significant, but the difference between the dup(15) autism and control group was significant ($p < 0.05$).

Subependymal Nodular Dysplasia

Nodular dysplasia was found in the brain of a 15-year-old adolescent girl diagnosed with dup(15) (Case 5). This consisted of a single large nodule in the wall of the temporal horn of the lateral ventricle and numerous nodules in the wall of the lateral ventricle in the occipital lobe. Subependymal nodular dysplasia was also detected in the brain of a 39-year-old woman diagnosed with dup(15) (Case 9; Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A323). There were numerous subependymal nodules less than 1 to 3 mm in diameter in the wall of the occipital horn of the lateral ventricle in a 32-year-old subject with idiopathic autism (Case 8; Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A324). These were composed of dysplastic neurons with a partially modified morphology of pyramidal, multipolar, or bipolar neurons and oval medium and small cells. In all 3 cases, the nodules were free of oval or polygonal giant cells or ballooned glial cells. Examination of the thalamus, caudate, putamen, nucleus accumbens, and globus pallidus did not reveal developmental qualitative abnormalities in these cohorts.

Differences Between the Global Pattern of Developmental Abnormalities in dup(15) and Autism

Although all dup(15) and subjects with autism had developmental abnormalities, the number of different types of developmental alterations detected in the brains of the dup(15) group was, on average, 2.3 times greater (6.9 per case; $n = 9$) than in the subjects with autism (3 per case; $n = 10$). Analysis of developmental alterations in 13 subjects with autism previously reported (49) revealed developmental abnormalities in the brains of all subjects with autism and a similar prevalence of alterations.

Other Neuropathologic Changes

Selective and marked neuronal loss without gliosis was found in the pyramidal layer in the CA1 sector in the brain of a 10-year-old boy with epilepsy (dup[15]; Case 2). Pathologic alterations extended from the head to the tail of the hippocampal formation, with loss of neurons in the range of 80% in the head and 50% in the body and tail. These findings might be the result of severe and frequent seizures.

An area of marked subpial gliosis was found within a sulcus between the inferior frontal and the orbital gyri in the brain of a dup(15) female with epilepsy and seizure-related asphyxia at the age of 26 years (Case 8). Almost complete focal loss of the granular layer was associated with gliosis, thickening of the affected molecular layer, degeneration of astrocytes, and deposition of many corpora amylacea. These findings most likely represent Chaslin gliosis, indicative of epilepsy-related brain damage. This pathologic condition coexisted with hyperconvolution of the DG, focal thinning, and duplication of the granular cell layer, considered developmental abnormalities contributing to abnormal electrical activity and seizures.

DISCUSSION

Knowledge of the clinical phenotype and genetic factors in autism is based on examination of thousands of individuals with idiopathic autism; however, between 1980 and 2003, only 58 brains of individuals with idiopathic autism were examined postmortem (50). Knowledge of the clinical and genetic characteristics of the dup(15) syndrome is based on examination between 1994 and 2006 of approximately 160 cases (47, 51–54), but the neuropathology of dup(15) with and without autism has not been studied. Results of the application of an extended neuropathologic protocol were previously reported for 13 brains of subjects with idiopathic autism (49). The current study characterizes qualitative neuropathologic changes in the brains of 9 individuals with

FIGURE 4. Cerebral cortex dysplasia autism spectrum disorder (ASD). **(A, B)** Polymicrogyria (**A**, arrowheads), focal hypocellularity or acellularity with lack of vertical and horizontal cortical organization (**B**, arrowhead) in an 8-year-old boy with ASD. **(C–F)** Bottom-of-a-sulcus dysplasia in a 5-year-old girl with idiopathic autism (Case 2). Focal dysplasia was limited to deep cortical layers **(C)**, affected all layers except the molecular layer **(D)**, and caused cortex fragmentation **(E)** or disruption of cortical ribbon continuity (**F**, arrowheads).

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dup(15), including 7 diagnosed as having an ASD (78%). This autism prevalence is in the highest range reported in clinical studies. The association with autism in some of the earlier individual reports (i.e. 4 [33%] of 12 [55] or 6 [36%] of 17) was not based on use of standardized screening (56). A standardized assessment of autistic manifestations in 29 children and adults with a supernumerary idic(15) detected in 20 individuals (69%) with a high probability of ASD (29). All studies reported a significant variability in the autistic phenotype, severity of autistic features, delayed development and/or ID, and seizures among subjects with dup(15) (15).

Major Neuropathologic Differences Between dup(15) Autism and Idiopathic Autism

Numerous studies indicate that autism is associated with a short period of increased brain size (57–59) and more neurons (60). Macrocephaly was detected in 37% of children with autism younger than 4 years (61) and in 42% of the 19 twins diagnosed with idiopathic autism younger than 16 years (6). Postmortem studies (62, 63) and imaging studies (64) also provided converging evidence of increased brain volume in autism. Microcephaly has been observed in only 15.1% of 126 children with autism aged 2 to 16 years (65) and is usually associated with severe pathology (66, 67), ID, and other medical disorders (65).

This study revealed a high prevalence of microcephaly in the dup(15) autism cohort examined postmortem, that is, the mean weight of the brains of subjects with dup(15) autism was 300 g less than that of subjects with idiopathic autism and 189 g less than that in the controls ($p < 0.001$ for both). The characteristics of head circumference and brain volume in dup(15) cohorts have been studied less comprehensively than in idiopathic autism, but published reports also show a strong prevalence of microcephaly. A summary of records from 107 supernumerary inv dup(15) cases revealed that only 3 subjects had macrocephaly (2.8%), but 6 times more cases (n = 18, 16.8%) had microcephaly (15). Battaglia (68) detected microcephaly in radiologic evaluations of 1 in 4 subjects with dup(15) whose age ranged from 4 to 8 years. These data suggest the failure of mechanisms controlling brain growth in autism, resulting in the prevalence of macrocephaly in idiopathic autism and of microcephaly in dup(15) autism.

Our extended neuropathologic protocol revealed several striking differences between the pattern of developmental alterations in dup(15) autism and idiopathic autism. Neuronal migration defects in the hippocampus resulting in heterotopias in the alveus, CA4, and DG were 8 times more common in dup(15) autism (89% of subjects) than in idiopathic autism (10%; $p < 0.001$). The second developmental abnormality distinguishing dup(15) autism from idiopathic autism was the dysplasia that occurred 8 times more often in the DG of subjects with dup(15) and the different types of developmental abnormalities that occurred 22 times more often in the DG of subjects with dup(15) ($p < 0.001$). The third factor differentiating these 2 cohorts was the absence of cerebral cortex dysplasia in dup(15) autism and the presence of this pathology in 50% of subjects with idiopathic autism ($p < 0.03$). The increased number of developmental alterations and the topographic differences suggest significant differences between mechanisms contributing to abnormal neuronal migration and altered cytoarchitecture in these 2 cohorts.

Linkage and gene mapping analysis, molecular reports, and clinical studies revealed the link between de novo, maternally derived proximal 15q chromosome alterations, and autism (13, 15, 27, 33, 56, 69–72). This postmortem study suggests that neuropathologic profile with microcephaly and multiple focal developmental defects is another marker of maternally derived proximal 15q chromosome alterations contributing to autistic phenotype.

Although ASD and epilepsy are heterogeneous disorders, they often occur together. This may indicate that these disorders share some underlying mechanisms and that epileptogenesis affects brain structure and function, which modify the clinical manifestations of autism. Approximately 30% of children with autism are diagnosed with epilepsy and 30% of children with epilepsy are diagnosed with autism (73). Significant cognitive impairment is present in approximately 50% of all individuals who have autism (74). Early onset of seizures contributes to clinical regression, enhanced severity of autistic phenotype, and enhanced mortality. The rate of death among subjects with autism is 5.6 times higher than expected (75), and epilepsy- and cognitive impairment–related accidents account for most of the deaths (75–78). The diagnosis of epilepsy in 78% (7/9) of postmortem-examined individuals with dup(15) indicates that epilepsy is an important component of the clinical phenotype in most individuals diagnosed with dup(15).

Microcephaly might be one of several indications of brain immaturity that increases the risk of epilepsy. The immature brain exhibits increased excitation, diminished inhibition, and increased propensity for seizures in infancy and early childhood (79). The reduced volume of neurons in most subcortical structures and some cortical regions in the brains of 4- to 8-year-old children with autism seems to reflect brain immaturity in early childhood (80). In the normally developing brain, maturation of the frontal and temporal cortex is associated with differential expression of 174 genes; however, none of these genes are differentially expressed in ASD (81). Altered development of neurons resulting in brain immaturity may contribute to an increased tendency for the seizures and epileptogenesis observed in the examined cohort. Very early onset of intractable epilepsy (at 10, 8, and 10 months, respectively) was reported in all 3 of the youngest individuals diagnosed with dup(15), who died as a result of SUDEP at 9, 10, and 11 years. These findings suggest that very early onset of seizures and very severe seizures may increase the risk of SUDEP in this cohort. Severe ID in all 3 of these individuals indicates that brain immaturity and a profound ID are another SUDEP risk factor in the examined cohort of subjects with dup(15). The combination of all these factors may contribute to death at a very early age (~10 years).

Sudden unexpected death in childhood (SUDC) is the sudden death of a child older than 1 year that, despite a review of the clinical history, circumstances of death, and complete autopsy with appropriate ancillary testing, remains unexplained.

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It occurs throughout childhood (<18 years) but occurs most commonly between the ages of 1 and 4 years (82, 83). Sudden unexpected death in childhood is an unexpected and unexplained death that occurs in patients with known epilepsy, including children, and is typically associated with sleep (84, 85). Kinney et al (86) reported several types of hippocampal anomalies in SUDC cases, including hyperconvolution of the DG, focal duplication of the DG, granular nodular heterotopia, abnormal folding of the subiculum, and focal clustering of pyramidal neurons in the cornu Ammonis. These developmental anomalies are considered a cause of seizure-related autonomic and/or respiratory dysfunction and sudden death (87–89). Kinney et al (86) proposed that these anomalies represent an epileptogenetic focus that, when triggered by fever, trivial infection or minor head trauma at a susceptible age, may result in unwitnessed seizure, cardiopulmonary arrest, and sudden death. The presence of these developmental anomalies in the examined brains of individuals with dup(15) may explain SUDEP in 5 cases and SUDC in 2 other cases. The presence of these changes in the hippocampus of subjects with dup(15) who died of causes other than SUDEP or SUDC suggests that they also were at higher risk for sudden unexpected death. Collectively, these data indicate that the risk of SUDC is much higher in the dup(15) cohort than in the general population with SUDC in which there is an overall rate of 57 of 100,000 deaths per year (90).

Microdysgenesis is not specific for dup(15), epilepsy, or autism; it has been reported in ID (91), psychosis (92), and dyslexia (93), as well as in some control subjects. None of these developmental alterations can be considered pathognomonic of an ''epileptic'' brain (83), but changes in the cohort affected by dup(15) or idiopathic autism reveal significant differences. This indicates that not a single lesion but, instead, a complex pattern of developmental defects distinguishes these subjects. A 2.3-fold higher prevalence of these developmental abnormalities, 2.3 times higher prevalence of epilepsy, and 6 times higher prevalence of epilepsy-related death in the dup(15) cohort compared with the idiopathic autism group suggest that the mechanisms leading to developmental structural alteration in the hippocampal formation are a major contributor to epilepsy and SUDEP/SUDC in dup(15). These seem to be a much weaker contributor to epilepsy and SUDEP in idiopathic autism. The results presented in this report reinforce the hypothesis that additional copies of the critical 15q11-q13 region are causally related to the autism phenotype and developmental abnormalities contributing to epilepsy and to an increased risk of SUDEP and SUDC. Future studies of the expression and distribution of proteins encoded by $GABA_A$ receptor subunit genes ($\alpha 5$, $\beta 3$, and $\gamma 3$) and the gene for juvenile epilepsy located near D14S165 on chromosome 15 may explain the role of duplication or triplication of these genes in autism and the enhanced susceptibility to seizures in dup(15) syndrome.

In conclusion, despite the common clinical diagnosis of autism in the dup(15) and idiopathic cohorts, significant differences in brain growth and focal developmental defects of neuronal migration and cytoarchitecture indicate that the dup(15) autistic phenotype is a product of unique genetic, molecular, and neuropathologic alterations.

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Abnormal Intracellular Accumulation and Extracellular Aβ Deposition in Idiopathic and Dup15q11.2-q13 Autism Spectrum Disorders

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Abstract

Background: It has been shown that amyloid ß (Aβ), a product of proteolytic cleavage of the amyloid β precursor protein (APP), accumulates in neuronal cytoplasm in non-affected individuals in a cell type–specific amount.

Methodology/Principal Findings: In the present study, we found that the percentage of amyloid-positive neurons increases in subjects diagnosed with idiopathic autism and subjects diagnosed with duplication 15q11.2-q13 (dup15) and autism spectrum disorder (ASD). In spite of interindividual differences within each examined group, levels of intraneuronal Aβ load were significantly greater in the dup(15) autism group than in either the control or the idiopathic autism group in 11 of 12 examined regions ($p<0.0001$ for all comparisons; Kruskall-Wallis test). In eight regions, intraneuronal Aβ load differed significantly between idiopathic autism and control groups ($p<0.0001$). The intraneuronal Aβ was mainly N-terminally truncated. Increased intraneuronal accumulation of $A\beta_{17-40/42}$ in children and adults suggests a life-long enhancement of APP processing with α-secretase in autistic subjects. Aβ accumulation in neuronal endosomes, autophagic vacuoles, Lamp1-positive lysosomes and lipofuscin, as revealed by confocal microscopy, indicates that products of enhanced α-secretase processing accumulate in organelles involved in proteolysis and storage of metabolic remnants. Diffuse plaques containing $A\beta_{1-40/42}$ detected in three subjects with ASD, 39 to 52 years of age, suggest that there is an age-associated risk of alterations of APP processing with an intraneuronal accumulation of a short form of Aβ and an extracellular deposition of full-length Aβ in nonfibrillar plaques.

Conclusions/Significance: The higher prevalence of excessive Aβ accumulation in neurons in individuals with early onset of intractable seizures, and with a high risk of sudden unexpected death in epilepsy in autistic subjects with dup(15) compared to subjects with idiopathic ASD, supports the concept of mechanistic and functional links between autism, epilepsy and alterations of APP processing leading to neuronal and astrocytic Aβ accumulation and diffuse plaque formation.

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Introduction

Autism is a developmental disorder characterized by qualitative impairments in reciprocal social interactions, verbal and nonverbal communication, and restricted, repetitive and stereotyped patterns of behavior [1]. Autism is often diagnosed in subjects with genetic disorders, including maternal origin duplications 15q11.2-q13

(dup15) (69%) [2,3], fragile X syndrome (FXS) (15–28%) [4] and Down syndrome (DS) (7%) [5].

Recent studies indicate that non-amyloidogenic cleavage of the amyloid-β (Aβ) peptide precursor (APP) with α and γ secretases is linked to several developmental disorders, including autism and FXS [6–10]. The proteolytic cleavage of APP by membrane-associated secretases releases several Aβ peptides possessing heterogeneous amino- and carboxyl-terminal residues, including $A\beta_{1-40}$ and $A\beta_{1-42}$ as products of β- and γ-secretases (amyloidogenic pathway); $A\beta_{17-40/42}$, as a product of α- and γ-secretases (p3 peptide, non-amyloidogenic pathway) [11,12]; and $A\beta_{pE3}$ as a product of N-terminal truncation of full-length Aβ peptide by aminopeptidase A and pyroglutamate modification [13]. Aβ peptides differ in toxicity, oligomerization, fibrillization, distribution and trafficking within cells, and in their contribution to Aβ deposits in plaques and vascular walls. Alzheimer disease (AD) is associated with oligomeric Aβ accumulation, fibrillar Aβ deposition in plaques, neuronal degeneration and cognitive decline. Intraneuronal Aβ accumulation has been shown to be an early event in AD brains and in transgenic mouse models of AD, and has been linked to synaptic pathology [14,15].

Detection of significantly increased levels of sAPP-α in blood plasma in 60% of autistic children was reported to be an early biomarker of a subgroup of children with autism [6]. Enhanced APP processing by α-secretase is especially prominent in autistic subjects with aggressive behavior [6,16]. Sokol et al. [10] proposed that increased levels of sAPP-α contribute to both the autistic and FXS phenotypes, and that excessively expressed sAPP-α neurotrophic activity may contribute to an abnormal acceleration of brain growth in autistic children and to macrocephaly in FXS. The fragile X mental retardation protein (FMRP) binds to and represses the dendritic translation of APP mRNA, and the absence of FMRP in FXS and in *Fmr1*^{KO} mice results in the upregulation of APP, $A\beta_{40}$ and $A\beta_{42}$ [7]. Westmark et al. [8] also revealed that genetic reduction of *AβPP* by removal of one *App* allele in *Fmr1*^{KO} mice results in reversion of FXS phenotypes, including reduction of plasma $A\beta_{1-42}$, to normal levels. Experimental studies in *Fmr1*^{KO} mice [17] suggest that over-expression of APP/Aβ may contribute to the seizures observed in autism [18] and FXS [4] and that both the over- and under-expression of APP and its metabolites increase the incidence of seizures [7,17,19,20].

Previously we reported that in the brains of controls, both children and adults, neurons accumulate cell type–specific amounts of $A\beta_{17-40/42}$, which is the product of nonamyloidogenic APP processing [21]. One may hypothesize that increased levels of sAPP-α in blood plasma [6,9,16] reflect the enhanced non-amyloidogenic processing of neuronal APP with α-secretase in the brain of autistic subjects.

The aims of this comparative study of the brains of subjects with idiopathic autism (autism of unknown etiology) and autism caused by maternal origin dup(15) were (a) to test the hypothesis that regardless of the causative mechanism, autism is associated with an enhanced accumulation of Aβ in neuronal cytoplasm, (b) to show that intraneuronal Aβ is the product of non-amyloidogenic α-secretase APP cleavage ($A\beta_{17-40/42}$), (c) to show brain region– and cell type–specific Aβ immunoreactivity, and (d) to identify cytoplasmic organelles involved in Aβ accumulation in the neurons of autistic and control subjects.

Results

The Difference between Intraneuronal Aβ Accumulation in dup(15) Autism, Idiopathic Autism and Control Groups

In all subjects with dup15/autism spectrum disorder (ASD) and the majority of individuals with idiopathic ASD, intraneuronal Aβ immunoreactivity was observed in more neurons, and the amount of immunoreactive material was increased in comparison to the control subjects (Fig. 1). The morphology of the intracellular deposits of Aβ-positive material was cell type–specific. Cortical pyramidal neurons showed significant heterogeneity of intraneuronal deposits with a mixture of fine granular material and several times larger 4G8-positive granules. In Purkinje cells, fine granular deposits were accumulated in the cell body. In the dentate nucleus, large neurons accumulated fine granular material, whereas small neurons accumulated a few much larger Aβ-positive granules. Neurons in the reticulate nucleus in the thalamus contained a mixture of fine granular material and large 4G8-positive granules.

Immunocytochemistry with monoclonal antibodies (mAbs) 4G8 (17–24 aa of Aβ) and 6E10 (4–13 aa of Aβ) revealed that almost all intraneuronal Aβ is 4G8-positive, but only a very small proportion is labeled with 6E10.

Quantitative evaluation of 12 brain subregions/cell types (frontal, temporal and occipital cortex, Purkinje cells, amygdala, thalamus, lateral geniculate body (LGB), dentate gyrus, CA1 and CA4 sectors and dentate nucleus) revealed that in 11 subregions intraneuronal Aβ load was significantly greater in the dup(15) autism group than in the control and idiopathic autism cohorts ($p<0.0001$ for all comparisons). In eight regions (all three cortical subregions, Purkinje cells, amygdala, thalamus, LGB, and dentate gyrus), intraneuronal Aβ load differed significantly between the idiopathic autism and control groups ($p<0.0001$). In structures with almost all neurons positive for Aβ–the dentate nucleus and the inferior olive–the amyloid load was insignificantly higher in control subjects than in subjects with idiopathic autism.

Quantitative study revealed different patterns of immunoreactivity in brain subregions (Fig. 2, and Supporting Information, Fig. S1). The characteristic feature distinguishing the amygdala, thalamus and Purkinje cells of subjects with dup(15) autism was the very high percentage of neurons with strong cytoplasmic Aβ immunoreactivity (46%, 46% and 35%, respectively); the percentage was significantly lower in the idiopathic autism group (32%, 38% and 19%, respectively), and very low in control subjects (6%, 6% and 12%, respectively). However, in pyramidal neurons in the frontal, temporal and occipital cortex, the percentage of neurons with strong Aβ immunoreactivity was low (3–10%), whereas the total percentage of Aβ-positive neurons was significantly higher in the dup(15) group (81–83%) than in the idiopathic autism group (56–71%) and in control subjects (45–51%).

The percentage of Aβ-positive neurons and neuronal amyloid load was smaller in the hippocampal formation, especially in the CA1 sector and dentate gyrus of control subjects. The amyloid load was significantly higher in the dup(15) autism group than in control subjects, but the difference in amyloid load between the idiopathic autism and control groups was insignificant (Fig. S1).

The feature distinguishing the LGB, inferior olive and dentate nucleus from other brain structures is the childhood onset of lipofuscin accumulation. In LGB, strong Aβ immunoreactivity was observed in 73% of neurons in dup(15) autism and in 62% in idiopathic autism but only 16% of LGB neurons were strongly Aβ-positive in control subjects. In the dentate nucleus, the percentage of strongly positive neurons was comparable in all three groups (41%, 35% and 41%, respectively), but overall amyloid load was statistically higher in dup(15) autism. The percentage of strongly Aβ-positive neurons in the inferior olive was the same in the idiopathic autism and in the dup(15) (32%) group, and there was no difference in overall amyloid load between autistic and control subjects (Fig. S1).

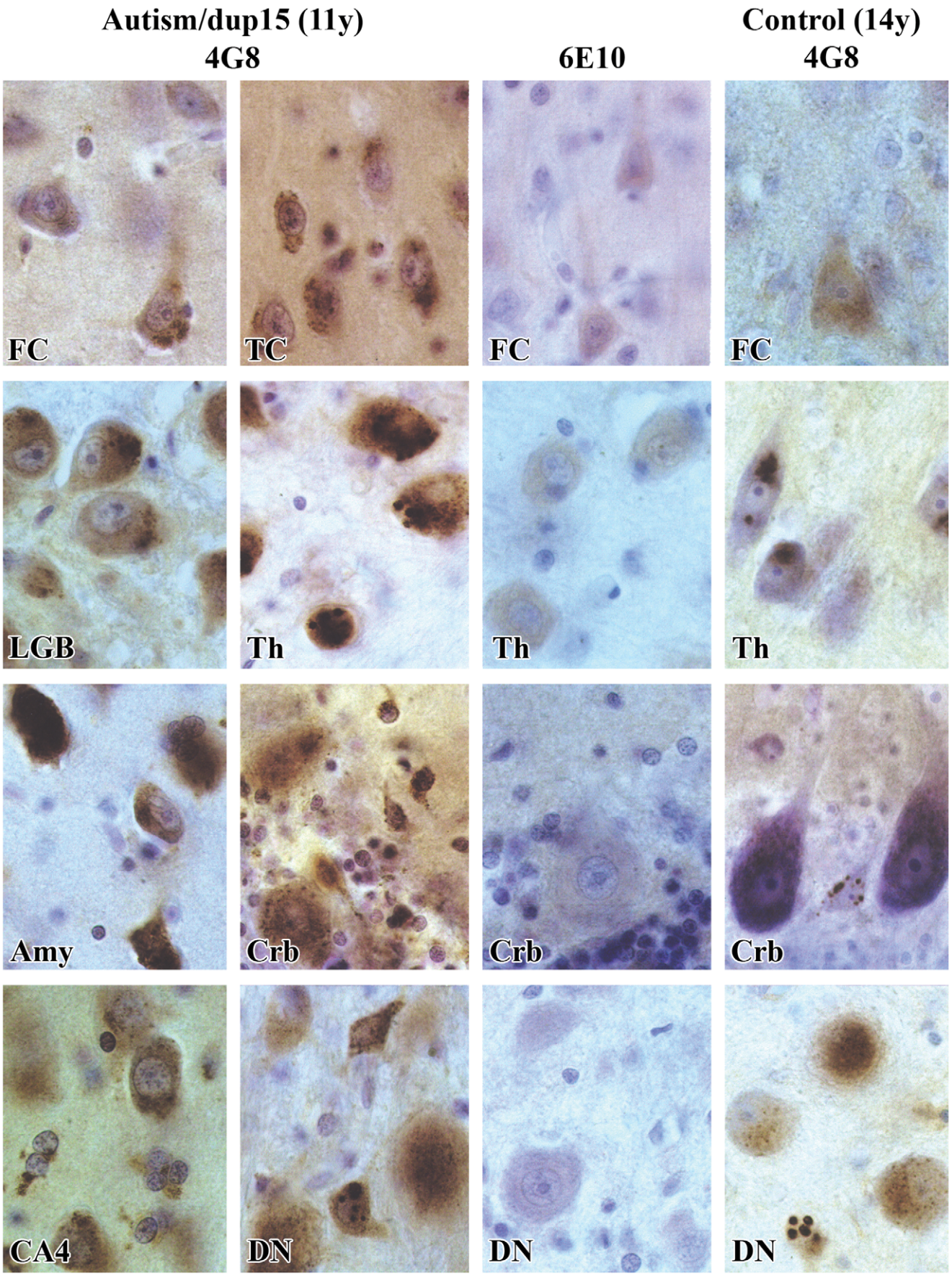

Autism/dup15 (11y)
4G8
6E10
Control (14y)
4G8
FC
TC
FC
FC
LGB
Th
Th
Th
Amy
Crb
Crb
Crb
CA4
DN
DN
DN

Figure 1. Enhanced intraneuronal accumulation of amino-terminally truncated Aβ in autism. Mapping of $A\beta_{17-24}$ in the brain AN09402 reveals brain region– and cell type–specific patterns of abnormal Aβ accumulation in the cytoplasm of neurons and glial cells of a male diagnosed with dup(15), autism and intractable epilepsy, whose sudden unexpected death at the age of 11 years was seizure-related. Almost all neurons in the frontal (FC) and temporal cortex (TC) are 4G8-positive, but the reaction intensity varies from weak to strong. Strong immunoreactivity is observed in many neurons in the lateral geniculate body (LGB), thalamus (Th), amygdala (Amy), Purkinje neurons and basket and stellate neurons in the molecular layer in the cerebellar (Crb) cortex, in many neurons and astrocytes in the CA4, and large and small neurons in the dentate nucleus (DN). Some types of neurons (in the reticular nucleus in the thalamus and small neurons in the dentate nucleus) have different types of deposits: fine-granular and 2- to 3-μm in diameter 4G8-positive deposits. No reaction or only traces of a reaction detected with mAb 6E10 in the frontal cortex, thalamus, cerebellum and dentate nucleus indicate that in intraneuronal Aβ the amino-terminal portion is missing, and the prevalent form of Aβ is α-secretase product. Immunoreactivity with mAb 4G8 is present in the brain of the control subject (14 years of age), but fewer neurons are positive, and immunoreactivity in the frontal cortex, thalamus, cerebellum and dentate nucleus is weaker than in the affected subject. In the control subject, glial cells are usually 4G8-immunonegative.
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Aβ in Glial Cells

Astrocytes and microglia in the control brains were usually Aβ-negative or contained only traces of Aβ immunoreactivity. Enhanced neuronal Aβ accumulation in the brains of individuals with autism was associated with Aβ accumulation in the astrocytes' cytoplasm and in some microglial cells (Fig. 3). Two patterns of Aβ immunoreactivity were observed in astroglia. The most common form was a condensed aggregate of Aβ in one pole of the astrocyte soma typical for CA4 sector, some cortical areas but without clear anatomical predilection, and the cerebellar cortex border zone between granule and molecular layers. The less common form was deposition of Aβ-immunoreactive granular material in the entire astrocyte body and in a proximal portion of processes radiating from the cell body (frequent in the molecular layer of the cerebral cortex). The increase in the amount of cytoplasmic Aβ was often paralleled by (a) a several-fold increase in the number of astrocytes, all of which were Aβ-positive (Fig. 3a), (b) clustering of astrocytes in groups of 3–10 cells (Fig. 3b), (c) numerous mitoses as a sign of astrocyte proliferation (Fig. 3c,d) and (d) astrocyte death resulting in deposition of extracellular remnants of Aβ aggregates (Fig. 3e) similar to those seen in astrocyte cytoplasm. Extracellular Aβ deposits were found in neuropil, but larger aggregates (more than 10) were more often in the perivascular space. Confocal microscopy confirmed Aβ accumulation in GFAP-positive astrocytes (Fig. 3, lower panel).

Intracellular Distribution of Amino-terminally Truncated Aβ in Neurons

Intraneuronal Aβ deposits revealed striking neuron type–specific differences in amount, morphology and cytoplasmic

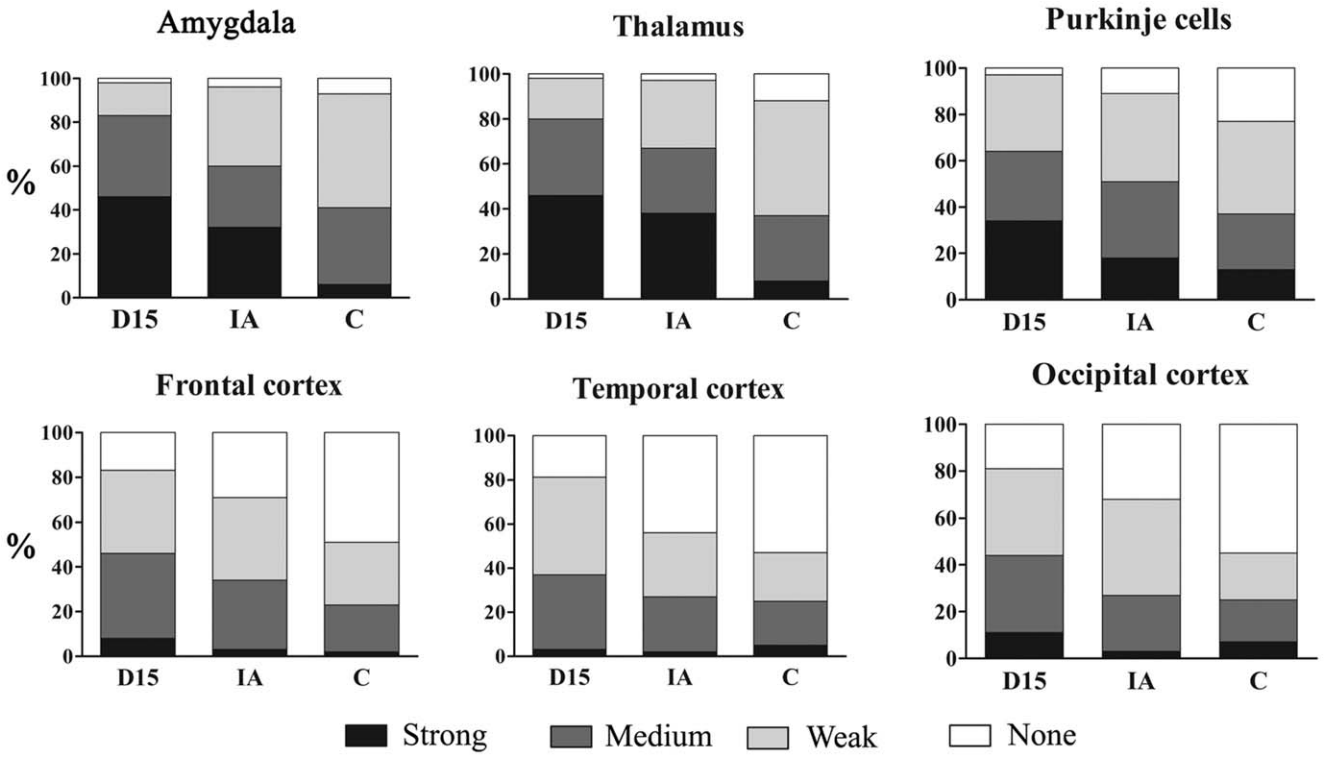


Figure 2. Two major patterns of alterations in intraneuronal Aβ accumulation. Graphs show a high percentage of neurons with strong cytoplasmic immunoreactivity (mAb 4G8) in the amygdala, thalamus and Purkinje cells in subjects diagnosed with dup(15) autism (D15), a lower percentage in idiopathic autism (IA) subjects, and a low percentage in control subjects. In contrast, the characteristic feature of pyramidal neurons in the frontal, temporal and occipital cortex is a low percentage of neurons with strong Aβ immunoreactivity, whereas the total percentage of Aβ-positive neurons is significantly higher in the dup(15) group than in the idiopathic autism group or in control subjects. Differences in Aβ immunoreactivity in the dup(15) autism vs. control cohort, the idiopathic autism vs. control group, and the dup(15) autism vs. idiopathic autism are significant ($p<0.0001$).
doi:10.1371/journal.pone.0035414.g002

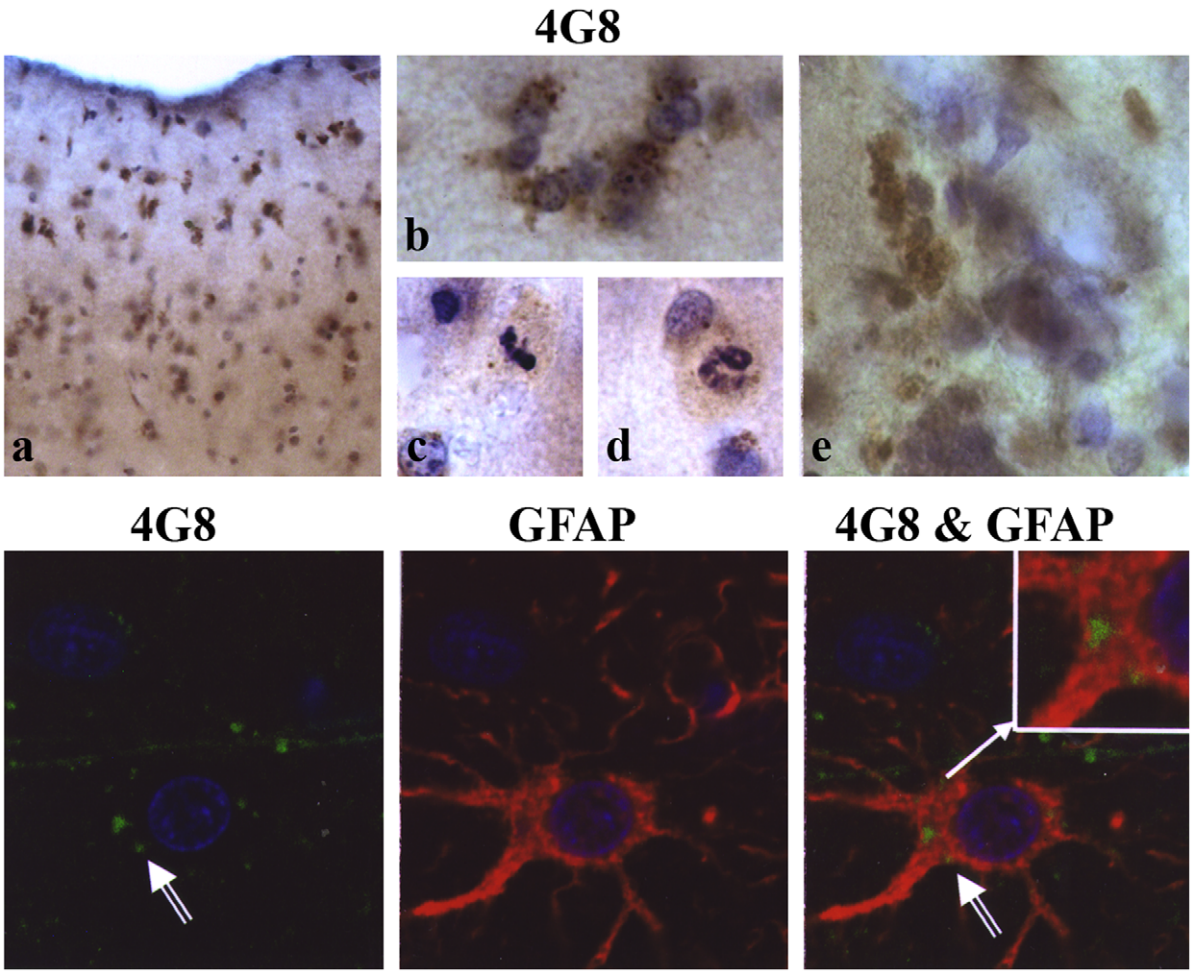


Figure 3. Enhanced accumulation of amino-terminally truncated Aβ in autistic subjects astrocytes. Clusters of 4G8-positive astrocytes, especially numerous in the molecular layer (a, b); very frequent mitotic divisions (c, d); and extracellular 4G8-positive Aβ deposits, with morphology of astrocytes' cytoplasmic aggregates (e) may reflect the enhanced proliferation, degeneration and death of Aβ-positive astrocytes in the brain of autistic subjects. Confocal microscopy confirmed the presence of Aβ (green; arrows) in the cytoplasm of GFAP-positive astrocytes (red). Cell nuclei were stained with TO-PRO-3-iodide (blue).
doi:10.1371/journal.pone.0035414.g003

distribution; however, they had the same immunoproperties. They revealed no reaction or traces of reaction with mAb 6E10 (Fig. 1) or 6F3D (not shown). The morphological diversity of Aβ deposits suggested that Aβ was present in different compartments of the endosomal-lysosomal pathway and in lipofuscin in neuron type–specific amounts. The number and size of Lamp1– (Fig. 4) lysosomes was from 2 to 3 times more than the number of Aβ-positive deposits; however, only about 10% of Aβ was detected in rab5-positive endosomal vesicles and in LC3B-positive autophagic vacuoles. Colocalization of Aβ with COXIV-positive mitochondria was observed in only a very few mitochondria.

Immunoreaction for Aβ detected with mAb 4G8 was present in some intracellular autofluorescent granules; however, the 4G8-immunoreactive deposits were detected also in neurons with scanty lipofuscin (Fig. 5) and in neurons with abundant autofluorescent granules. On the other hand, some neurons with scanty immunoreaction for Aβ contained numerous autofluorescent granules. The autofluorescent granules were not immunostained with mAb 6E10. Immunoreaction with polyclonal antibody (pAb) R226, specific for the C-terminus of Aβ42, showed only a fraction of labeling colocalized with autofluorescent granules. These results indicate that the detected intraneuronal immunostaining reflects accumulation of N-terminally truncated Aβ in several cellular compartments, including lipofuscin granules.

Specificity of Immunohistochemical Detection of Aβ with mAb 4G8 and 6E10

The epitopes of mAbs 6E10 and 4G8 (4–13 aa and 17–24 aa of the Aβ sequence, respectively) are present in full-length APP and APP C-terminal fragments. In brain tissue that has been fixed in formalin for several months, embedded in polyethylene glycol (PEG) and pretreated with 70% formic acid for 20 min, the immunostaining with mAb 4G8 (Fig. 6) and with 6E10 and 7F3D (8–17 aa of Aβ; not shown) is consistent with the distribution and amount of Aβ, but different from the distribution and amount of neuronal APP. In control brains, antibody R57 detects abundant intraneuronal APP immunoreactivity, but mAb 4G8 reveals only a very limited reaction with Aβ. In numerous neuronal populations in autistic subjects, the immunoreactivity for Aβ increases very significantly, but most R57 immunoreactive material is 4G8-negative, and most 4G8-positive granules are negative for APP. These results indicate that in the examined material, mAbs 6E10,

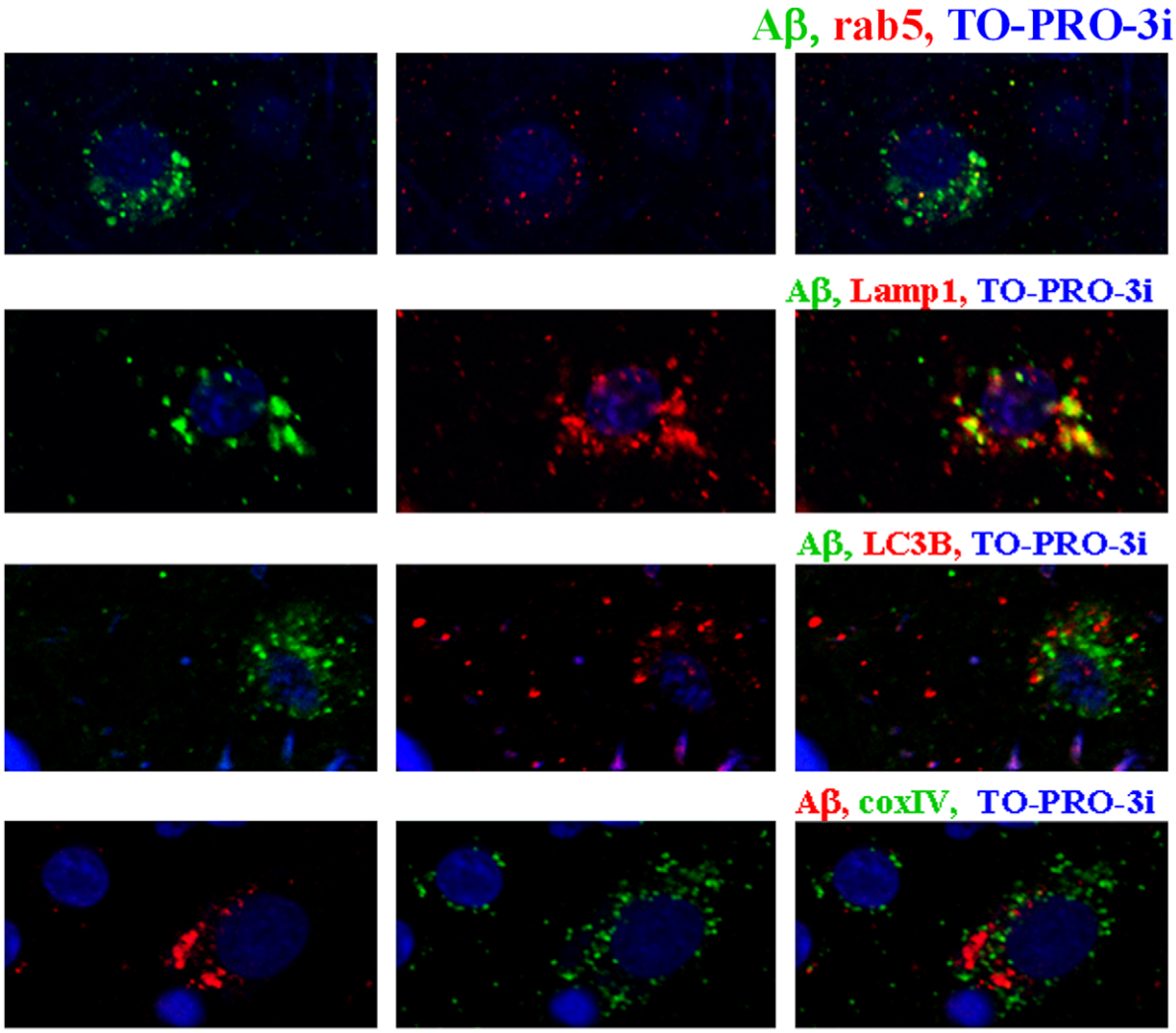


Figure 4. Aβ in endocytic vesicles, autophagic vacuoles, lysosomes and mitochondria. Co-localization of Aβ (4G8) in neurons in the frontal cortex of a 10-year-old subject diagnosed with autism/dup(15) (AN06365) demonstrates that a small portion of cytoplasmic Aβ is stored in rab5-positive endocytic vesicles and LC3B-positive autophagic vacuoles, whereas the largest proportion of Aβ is colocalized with lysosomal Lamp1. Colocalization of a relatively large portion of cytoplasmic Aβ with lysosomal markers appears to reflect the accumulation of products of intracellular degradation of Aβ that originated from endocytic and autophagic pathways. The presence of only a few Aβ-positive mitochondria immunolabeled with COXIV may suggest that this Aβ makes the smallest contribution to the detected neuronal Aβ accumulation and degradation pathway.
doi:10.1371/journal.pone.0035414.g004

4G8 and 7F3D detect Aβ but do not bind to neuronal APP detected with pAb R57.

Diffuse Plaque Distribution and Immunoproperties in the Brain of Autistic Subjects

Aβ-positive plaques were detected in one of the nine examined subjects diagnosed with dup15 (AN11931), and in two of the 11 subjects diagnosed with idiopathic autism (AN17254 and BB1376). All three subjects were the oldest in each group. In the dup(15) group, a 39-year-old female with autistic features and intractable epilepsy (onset at 9 years of age) and whose death was epilepsy-related had clusters of plaques in several neocortical regions, including the frontal, temporal and insular cortex (Fig. 7). Plaques were also found in the brains of two individuals diagnosed with idiopathic autism, including a 51-year-old subject who had had only one grand mal seizure (Fig. 8), and a 52-year-old individual whose records do not contain information about epilepsy or brain trauma. In both brains, the postmortem examination revealed numerous plaques within the entire cortical ribbon (Fig. S2) and in the amygdala, thalamus and subiculum (not shown).

In all three cases, thioflavin S staining did not reveal fluorescence in the plaques (not shown), suggesting that the amyloid plaques detected in the examined subjects with autism/dup(15) and idiopathic autism were nonfibrillar. However, positive immunoreactivity with all six antibodies used, including 6E10, 6F3, 4G8, Rabm38, Rabm40 and Rabm42 (Fig. 7 and 8) and 6F3D (not shown), revealed full-length $A\beta_{1-40/42}$ peptides. In the plaque area, numerous glial cells, mainly with the morphology of astrocytes, and less numerous, glial cells with the morphology of microglial cells, contained Aβ-immunoreactive granular material. In contrast to the presence of full-length Aβ peptides in plaques, the Aβ peptides in both astrocytes and microglial cells in the plaque perimeter and surrounding tissue were mAb 6E10- and 6F3D-negative, indicating that they were the product of α-secretase. They were positive for the three other antibodies,

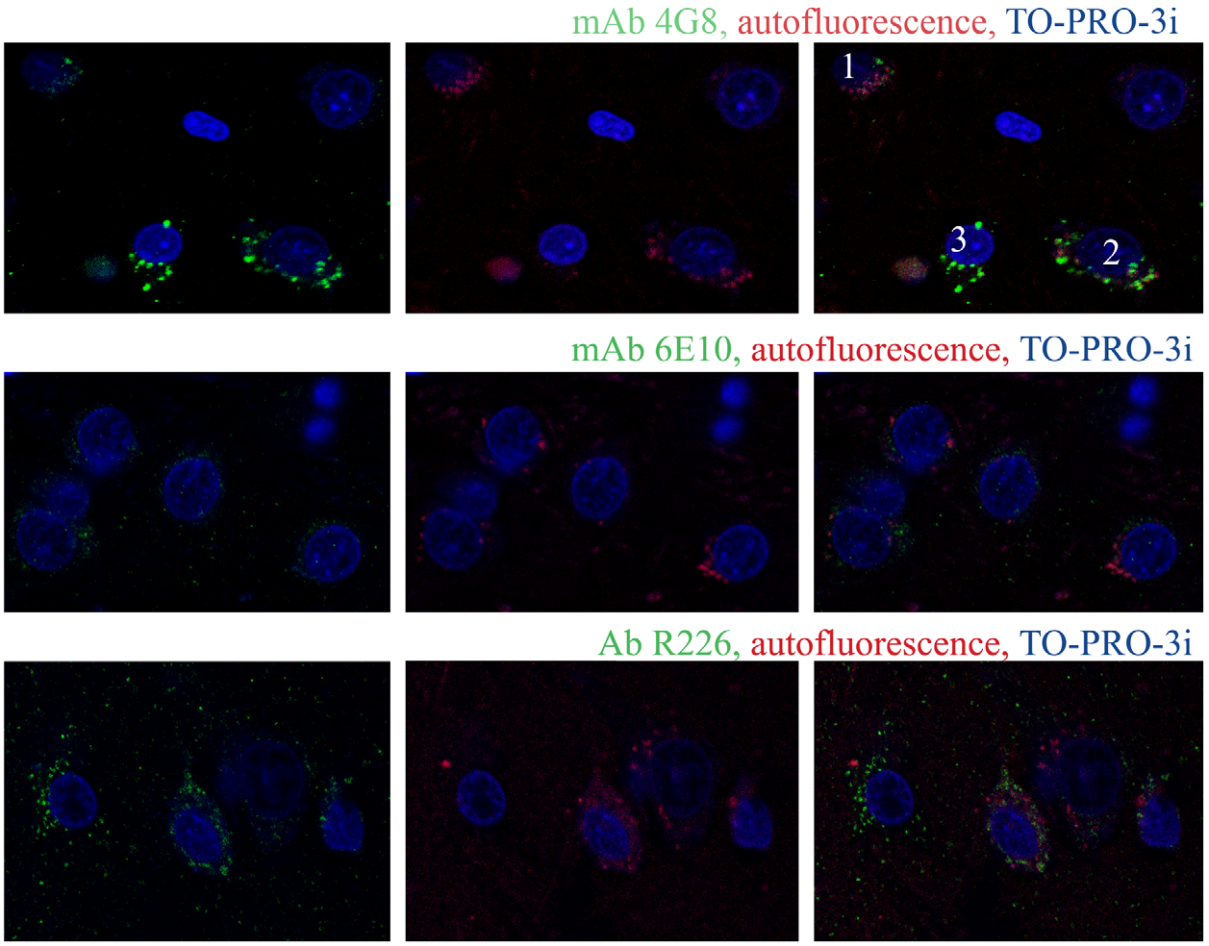


Figure 5. Aβ in lipofuscin. Accumulation of Aβ in lipofuscin in the frontal cortex of a 10-year-old subject diagnosed with dup(15) autism was characterized using mAbs 4G8 and 6E10 and pAb R226. Autofluorescent lipofuscin granules were 4G8-negative (cell 1) or partially positive (cell 2), but Aβ was also accumulated in lipofuscin-free neurons (cell 3). The neurons revealed only traces of reaction with mAb 6E10 and a moderate amount of pAb R226–positive Aβ42, which was partially co-localized with autofluorescent lipofuscin.
doi:10.1371/journal.pone.0035414.g005

Rabm38, Rabm40 and Rabm42, demonstrating that both astrocytes and microglia accumulate $A\beta_{17-40/42}$.

The extracts from the areas of the cerebral cortex in which diffuse plaques were detected by immunohistochemistry contained Aβ, mainly Aβ1–42, revealed by immunoblotting as a 4-kD band reacting with pAb R226 and mAb 6E10. The levels of Aβ1–42 in the samples exceeded 1.5 fmol per 1 μg of extracted proteins, whereas the levels of extracted Aβ 1–40 were low, below 0.2 fmol per 1 μg of extracted proteins (Fig. 9).

Immunoblotting of lysates from the cerebral cortex of autistic subjects without plaques and age-matched control subjects detected Aβ42 (Fig. 10) and Aβ40 (not shown) as a 3- to 4-kD band reacting with the pAb R226 and pAb R162, respectively. The levels of Aβ42 in the samples were in the range below 0.5 fmol per 40 μg of total proteins.

Neurofibrillary Degeneration

A very few neurofibrillary tangles (NFTs) were found in the entorhinal cortex and amygdala in a 43-year-old control subject and in the entorhinal cortex and cornu Ammonis of a 47-year-old control subject. A few NFTs were found in the entorhinal cortex, CA1 and parasubiculum in a 51-year-old autistic subject and in the entorhinal and temporal cortex and the amygdala of a 52-year-old autistic subject. Neurofibrillary changes were not found in the dup(15) autism cohort with the oldest examined subject who died at the age of 39 years.

Discussion

The accumulation of intraneuronal Aβ is considered a first step leading to amyloid plaque formation in AD [14,22–24]. However, our examination of control brains during the life span showed that intraneuronal Aβ also occurs in normal controls and that almost all cytoplasmic Aβ peptides are the product of α- and γ-secretases ($A\beta_{17-40/42}$) [21], whereas, the majority of amyloid in plaques is the product of β- and γ-secretases. This finding suggests that brain region– and neuron type–specific patterns of intraneuronal $A\beta_{17-40/42}$ peptide accumulation in control brains are a baseline for detection and evaluation of increases associated with autism, FXS, epilepsy, brain trauma or age-associated neurodegeneration, such as AD.

Detection of Aβ in Human Postmortem Material

The epitopes of mAbs 6E10 (4–13 aa of the Aβ sequence) and 4G8 (17–24 aa) are present in full-length APP and various APP fragments. Recently, Winton et al. [25] demonstrated that neuronal APP is immunolabelled with these two antibodies in

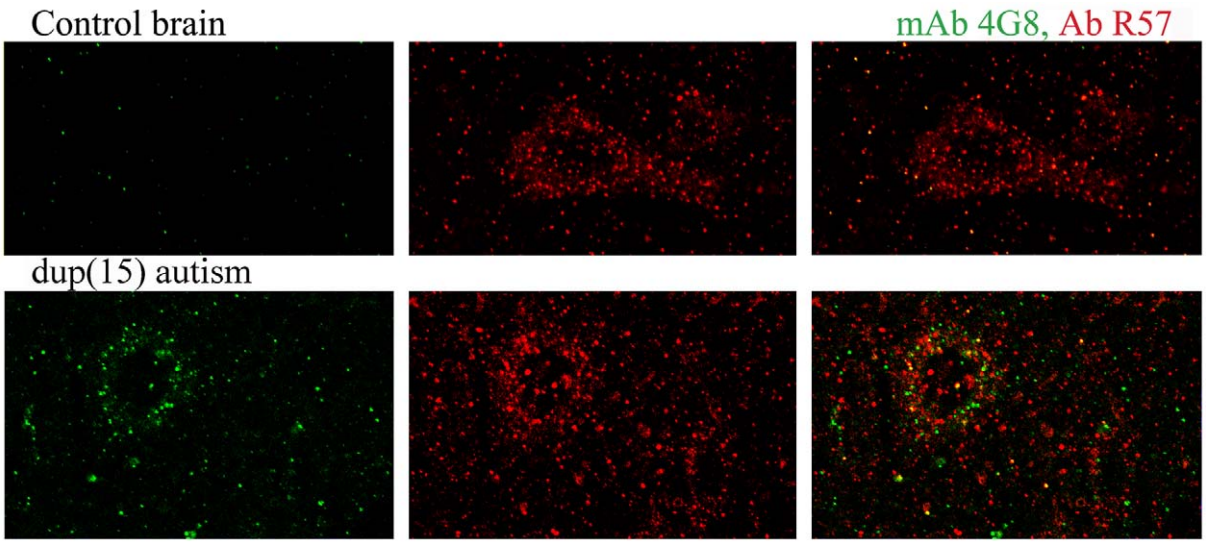


Figure 6. Immunoreactivity of mAb 4G8 with Aβ. mAb4G8 detects Aβ but does not detect APP in immunohistochemical staining in formalin-fixed and PEG-embedded samples of the frontal cortex of an 8-year-old control subject and a 10-year-old subject diagnosed with dup(15) and autism. Neurons in the control brain contain numerous granules that are immunoreactive with C-terminal APP–specific pAb R57 and are 4G8 negative. In the neurons of an autistic subject, only a few very numerous 4G8-positive deposits are R57-positive, whereas the majority of very numerous APP-immunoreactive granules are 4G8-negative.
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mouse brain fixed for 24 hours in 10% neutral buffered formalin. However, the pattern of immunostaining in human brain fixed in formalin for at least several months, dehydrated almost 3 weeks in ascending concentrations of ETOH, and embedded in PEG indicates that mAbs 4G8, 6E10 and 6F3D do not detect APP in tissue subjected to this process. The role of technical factors in the loss of access of these antibodies to their epitopes in APP was previously documented in studies of tissue fixed in formalin for 10 days and in studies of cultured cells [26,27]. Several observations in this report indicate that these antibodies do not detect APP. Massive immunolabelling of neuronal APP with R57 is in striking contrast with the presence of only traces of 6E10 and 6F3D immunoreactivity in these cells and the only partial co-localization of Aβ and APP labeling in the amyloid-rich neurons of autistic subjects. These data indicate that in the examined material, APP is detected with the APP-specific antibody R57, but not with mAbs 4G8, 6E10,and 6F3D, which, however, detect Aβ. One may assume that the epitopes of these antibodies, but not the R57 epitopes, are blocked or modified in APP molecules during long exposure to chemicals used for fixation, dehydration and embedding. Consistent with immunocytochemistry, Western blotting identifies 3–4 kD Aβ not only in subjects with diffuse plaques, but also in autistic subjects without plaques and in control subjects.

Excessive Accumulation of $A\beta_{17-24}$ in Neurons in Idiopathic Autism and dup(15) Autism

This is the first report documenting excessive accumulation of Aβ in the neurons of subjects with idiopathic autism and an even more pronounced accumulation in the dup(15) autism cohort. Two patterns of excessive accumulation distinguish these two cohorts from control subjects and indicate that excessive accumulation is neuron type/brain region–specific. Type 1 of altered Aβ accumulation is reflected in an increase in the percentage of neurons with strong Aβ accumulation by 7.6-fold in the amygdala and thalamus and by 4.5-fold in the LGB in individuals with dup(15) autism in comparison to the control group. A similar (by 5.3×, 6.3× and 3.9×, respectively) and statistically significant increase was found in the idiopathic autism group. Type 2 of altered Aβ accumulation is reflected in a more uniform increase in the percentage of neurons with combined strong, moderate and weak immunoreactivity. Again, this pattern is observed in both autistic cohorts in the pyramidal neurons in all three examined cortical regions.

These findings suggest that metabolic alterations are similar in both types of autism and that the severity of these alterations is less pronounced in idiopathic autism than in autism caused by dup(15). The significant increase in the percentage of neurons with enhanced cytoplasmic Aβ load in idiopathic autism and the fact that almost all of this Aβ is the product of α-secretase show the striking similarity to increased levels of sAPP-α in blood plasma in 60% of autistic children (6,16). In studies by Sokol et al. [6] and Ray et al. [16], aggressive behavior was identified as associated with increased levels of sAPP-α. Bailey et al. [9] also detected a significant increase in sAPP-α levels in 60% of autistic children but with no association between the severity of aggression, social or communication sub-scores and increased levels of sAPP-α. Due to the neurotrophic properties of sAPP-α, the authors proposed that an increased level of the products of α-secretase may help identify a subset of children in which early regional brain overgrowth is necessary and sufficient for the development of autism and may even represent a mechanism regulating overgrowth in autism. However, the most pronounced accumulation of amino-terminally truncated Aβ observed in the dup(15) autism cohort with microcephaly [28] indicates that intraneuronal Aβ accumulation of the products of α-secretase is not associated with brain overgrowth. Our data identify a dup(15) autism subcohort with microcephaly, more severe clinical phenotype, very early onset of seizures, a high percentage of intractable seizures, and a high prevalence of sudden unexpected death in epilepsy (SUDEP) as associated with the highest percentage of neurons accumulating α-secretase product.

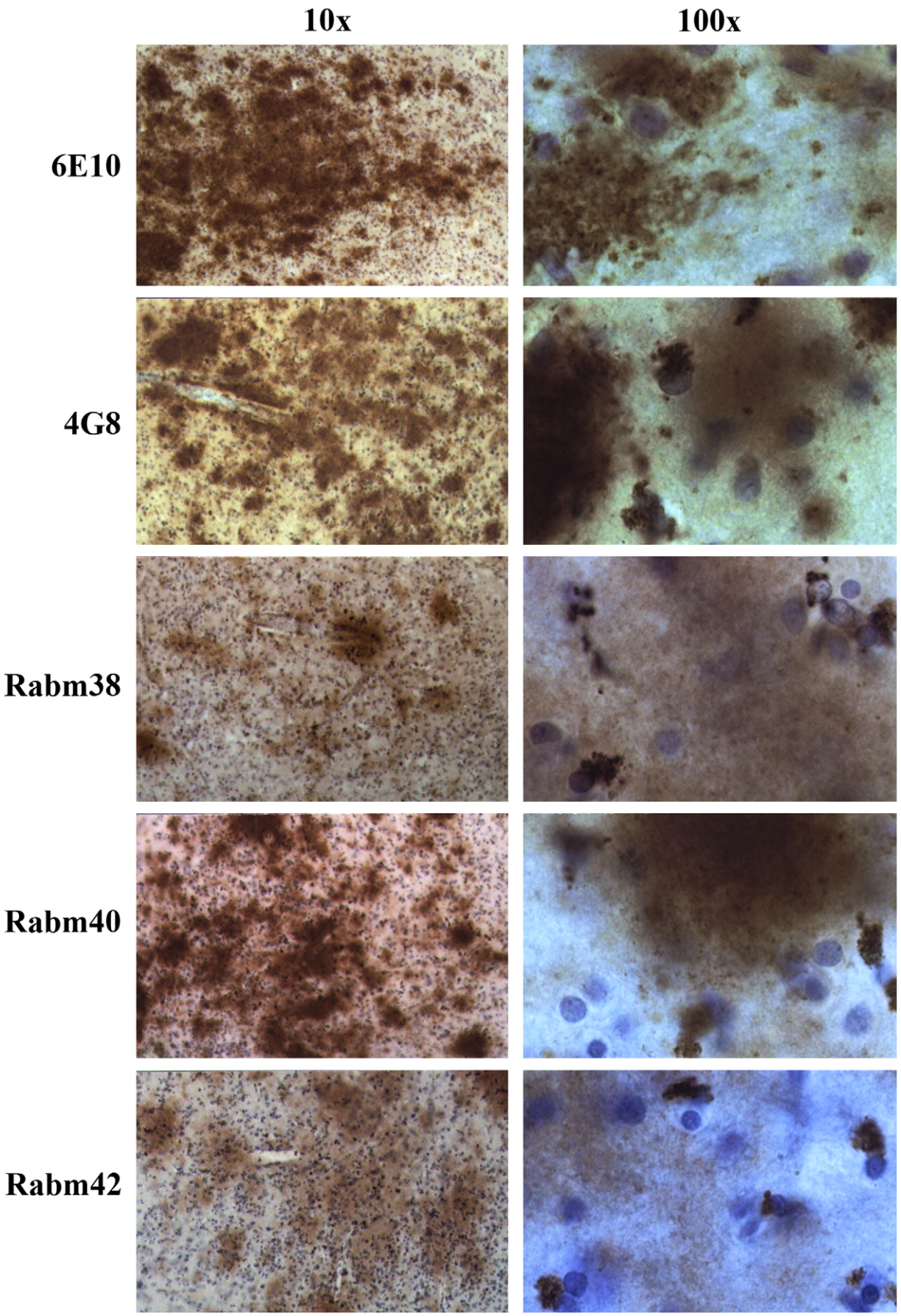

10x
100x
6E10
4G8
Rabm38
Rabm40
Rabm42

Figure 7. Full-length Aβ in diffuse plaques and amino-terminally truncated Aβ in astrocytes in autism/dup15. Diffuse plaques in the frontal cortex of a 39-year-old female (AN11931) diagnosed with dup(15), autistic features, and intractable seizures (age of onset 9 years) and whose death was epilepsy-related, are 6E10-, 4G8-, Rabm38-, Rabm40- and Rabm42-positive. Reaction with Rabm38 and Rabm42 was weaker than with other antibodies. Almost all glial cells with the morphology of astrocytes detected in the plaque perimeter had a large cluster of granular material located usually at one cell pole and positive with all antibodies detecting Aβ, except 6E10.
doi:10.1371/journal.pone.0035414.g007

Trafficking of $A\beta_{17–24}$ in Neurons

Aβ is generated in the endolysosomal pathway and in the endoplasmic reticulum/Golgi compartment [29–33] and is also detected in multivesicular bodies [34] and in mitochondria [15,35]. The application of Lamp1 as a lysosomal marker revealed that approximately 20–30% of neuron cytoplasmic $A\beta_{17–24}$ accumulates in this step of the proteolytic pathway in control and autistic subjects. An increase in cathepsin D protein expression, as reported in several brain regions of autistic subjects, suggests the selective enhancement of target proteins' hydrolysis by this aspartic acid protease [36]. The lysosome is the major acid hydroxylase-containing cell compartment engaged in processing of substrates delivered by (a) endocytosis, (b) autophagy [37] and (c) scavenging of proteins from the endoplasmic reticulum to lysosomes [38]. The increase of $A\beta_{17–24}$ in the lysosomes of autistic subjects may reflect $A\beta_{17–40/42}$ generation in these pathways.

This study revealed that another 20–30% of neuron $A\beta_{17–40/42}$ is present in lipofuscin, which is the final product of cytoplasmic proteolytic degradation of exogenous and endogenous substrates. During the entire lifespan, lipofuscin gradually accumulates in neurons [39]. The age of onset and dynamics of lipofuscin deposition are cell type–specific [40,41]. Our previous study revealed that neurons in the inferior olive, dentate nucleus and lateral geniculate body start accumulating lipofuscin and $A\beta_{17–40/42}$ early in life and that this accumulation progresses with age at region-specific rates [21]. The confocal microscopy study indicates that in spite of the known nonspecific binding of some antibodies to lipofuscin, the selection of the immunostaining protocol and the setting of proper thresholds in confocal imaging applied in this study reveal the selectivity of mAbs 4G8 and 6E10, and pAb R226 binding to some lipofuscin deposits.

The pattern of both Aβ and lipofuscin accumulation can be modified in early childhood in subjects with autism and even more significantly in individuals with dup(15) autism. The difference is detectable as an increase in the percentage of $A\beta_{17–40/42}$ immunoreactive neurons, the amount of immunopositive material per neuron, and the number of brain regions and neuron types affected in both children and adults. Detected changes in Aβ accumulation may reflect abnormal accumulation of lipofuscin, as reported by Lopez-Hurtado and Prieto [42]. An increase in the number of lipofuscin-containing neurons by 69% in Brodmann area (BA) 22, by 149% in BA 39, and by 45% in BA 44, in brain tissue samples from autistic individuals 7 to 14 years of age, was observed together with a loss of neurons and glial proliferation. However, enhanced lipofuscin accumulation is not unique for idiopathic autism or autism/dup(15). It has been reported in Rett syndrome [43], an ASD, as well as in several psychiatric disorders, including bipolar affective disorder [44] and schizophrenia [45,46].

Enhanced lipofuscin accumulation and enhanced $A\beta_{17–40/42}$ immunoreactivity in the majority of the examined brain structures in most of the individuals with autism and the subjects with dup(15) may be a reflection of enhanced oxidative stress. Oxidative stress contributes to protein and lipid damage in cytoplasmic components, their degradation in lysosomal and autosomal pathways, and the deposition of products of degradation in lipofuscin or their exocytosis [47,48]. The link between oxidative stress, cytoplasmic degradation and lipofuscin deposition is supported by the presence of oxidatively modified proteins and lipids in lipofuscin [39,49,50]. A significant increase in malondialdehyde levels (a marker of lipid peroxidation) in the plasma of autistic children [51] and in the cerebral cortex and cerebellum [52] may reflect oxidative damage leading to enhanced degradation, and the possible increased turnover of affected cell components.

Biological Activity of N-terminally Truncated Aβ

The results of confocal microscopy suggest that on average, 30% of neuronal Aβ is present in lysosomes and another 30% in lipofuscin. However, the biological consequences of accumulation of Aβ, in the lysosomes or in lipofuscin are not known. N-terminally truncated Aβ peptides exhibit enhanced peptide aggregation relative to the full-length species [53] and retain their neurotoxicity and β-sheet structure. Soluble intracellular oligomeric Aβ (oAβ) species inhibit fast axonal transport (FAT) in both anterograde and retrograde directions [54]. Inhibition of FAT results from activation of endogenous casein kinase 2. Altered regulation of FAT markedly reduces transport of synaptic proteins and mitochondria in the AD brain and in AD mouse models that accumulate oAβ [55]. Dysregulation of FAT results in distal axonopathies with a reduced delivery of critical synaptic elements required for the integrity, maintenance and function of synapses [54].

The *in vitro* studies suggest that Aβ 17–24 is toxic to neurons. Treatment of SH-SY5Y and IMR-32 human neuroblastoma cells with Aβ 17–24 causes apoptotic death similar to in cells incubated with Aβ1–42, whereas treatment with Aβ17–40 results in a lower level of apoptosis, comparable to experimental exposure to Aβ1–40. This apoptosis is mediated predominantly by the caspase-8 and caspase-3 pathways [56]. However, *in vitro* studies of the neuronal response to exogenous Aβ peptides do not replicate the neuronal exposure to endogenous $A\beta_{17–40/42}$ trafficking inside vesicles and vacuoles of lysosomal pathway.

$A\beta_{1–40/42}$ in Diffuse Plaques of Autistic Subjects

The presence of diffuse nonfibrillar plaques in two autistic subjects who were more than 50 years old and in one 39-year-old subject with autism/dup(15) suggests that in the fourth/fifth decade of life, there is an increased risk of the second type of changes: activation of the amyloidogenic pathway of APP processing with β- and γ-secretases, resulting in focal deposition of $A\beta_{1–40/42}$ in plaques. It was hypothesized that $A\beta_{17–42}$ peptides may initiate and/or accelerate plaque formation, perhaps by acting as nucleation centers that seed the subsequent deposition of relatively less amyloidogenic but apparently more abundant full-length Aβ [53,57,58]. Gouras et al. [59] considered intracellular $A\beta_{42}$ accumulation an early event leading to neuronal dysfunction. The $A\beta_{1–40/42}$ –positive diffuse plaques in the brains of autistic subjects are different from the $A\beta_{17–40/42}$–positive cerebellar diffuse plaques detected in DS [57,60]. Diffuse amorphous nonfibrillar Aβ deposits, called amorphous plaques [61], pre-plaques [62] or pre-amyloid deposits [63], are considered to be of neuronal origin [64–67]

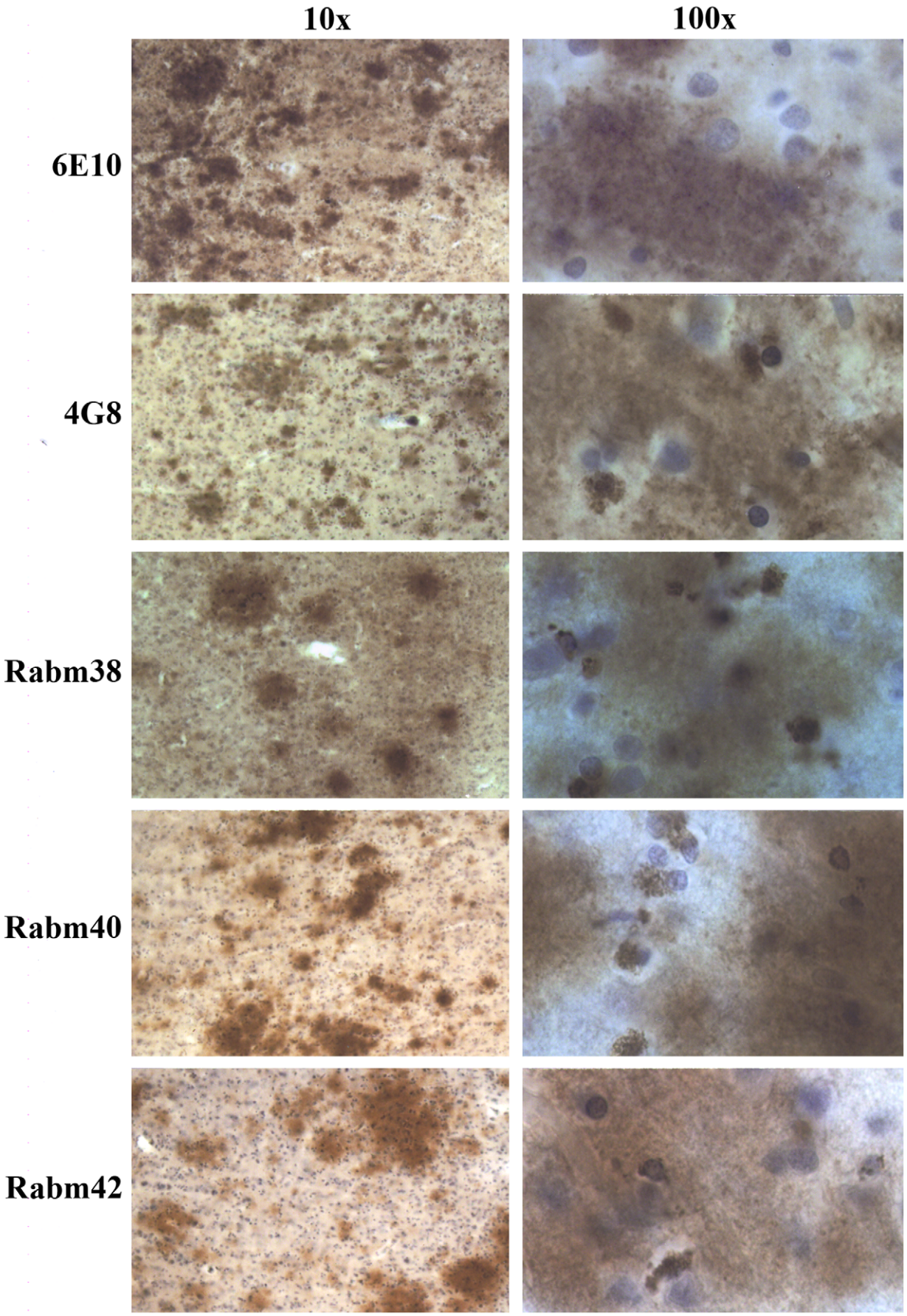

10x
100x
6E10
4G8
Rabm38
Rabm40
Rabm42

Figure 8. Full-length Aβ in diffuse plaques, and truncated Aβ in astrocytes in idiopathic autism. Diffuse plaques in the frontal cortex of a 51-year-old subject (AN17254) diagnosed with idiopathic autism, who had had only one grand mal seizure and died because of cardiac arrest, are immunopositive when stained with all five antibodies (6E10, 4G8, Rabm38, Rabm 40 and Rabm 42), but granular material in the cytoplasm of glial cells is immunopositive for all antibodies used except 6E10.
doi:10.1371/journal.pone.0035414.g008

and are formed selectively in projection areas of distant affected neuronal populations [68]. Diffuse plaque formation in autistic subjects suggests the activation of the secretory pathway and the synaptic release of $A\beta_{1-40/42}$.

The presence of $A\beta_{17-40/42}$ in astrocytes in $A\beta_{1-40/42}$ –positive diffuse plaques suggests that the full-length Aβ released by neurons is phagocytosed and processed by local astrocytes. One may hypothesize that the proliferation of Aβ-positive astrocytes, the increase of cytoplasmic Aβ immunorectivity in astrocytes, the presence of Aβ in all astrocytes in the affected region, astrocyte death and the deposition of large aggregates of extracellular Aβ in the cerebral cortex or hippocampus of autistic children and young adults is a response to the elevated levels of extracellular $A\beta_{17-40/42}$ and/or $A\beta_{1-40/42}$. Therefore, the number of Aβ-positive astrocytes may be an indicator of the local concentration of extracellular Aβ not only in plaque-positive but also in plaque-negative brain regions, occurring decades before plaque formation. Cytoplasmic granular immunoreactivity (Aβ17–23 and Aβ8–17) was reported in astrocytes in AD [69]. In astrocytes, intracellular Aβ appears in lysosomes and lipofuscin [70,71]. It defines the role of astrocytes in the uptake of different species of Aβ in diffuse and neuritic plaques and their subsequent degradation in lysosomes and storage of products of degradation in lipofuscin [69].

In the examined autistic cohort, the early onset of intractable epilepsy and the epilepsy-related chronic and acute brain trauma appear to be additional risk factors for APP pathway activation and diffuse plaques formation. Repetitive brain trauma, including that related to epilepsy and head banging, produces a chronic traumatic encephalopathy with the associated deposition of Aβ, most commonly as diffuse plaques [72–74]. In acute traumatic brain injury, diffuse cortical Aβ deposits were detected in 30% to 38% of cases 2 hours after injury [75–77].

The presence of a few NFTs in the entorhinal cortex, cornu Ammonis and amygdala in 43- and 47-year-old control subjects and in these structures and in the parasubiculum and temporal cortex of 51- and 52-year-old autistic subjects is consistent with the topography and amount of age-associated neurofibrillary degeneration and NFT distribution observed in the general population [78].

In conclusion, this postmortem study of Aβ distribution in the brain of subjects with idiopathic autism and dup(15) autism suggests (a) very significant enhancement of intraneuronal Aβ accumulation in almost all examined cortical and subcortical structures in autism, especially in autism associated with dup(15); (b) the prevalence of anabolic α-secretase APP processing and $A\beta_{17-40/42}$ accumulation in neuronal endosomes, autophagic vacuoles, lysosomes and lipofuscin in the majority of autistic children and adults; and (c) activation of the amyloidogenic pathway of APP processing with β- and γ-secretases in the late adulthood of some autistic subjects with diffuse nonfibrillar plaque formation and astrocyte activation.

Materials and Methods

Material, Clinical and Genetic Evaluation

The brains studied were from nine individuals diagnosed with dup(15) ages 9 to 39 years (five males and four females), 11 subjects with idiopathic autism ages 2 to 52 years (10 males and one female), and eight control subjects ages 8 to 47 years (four males and four males) (Table 1). Medical records were obtained following consent for release of information from the subjects' legal

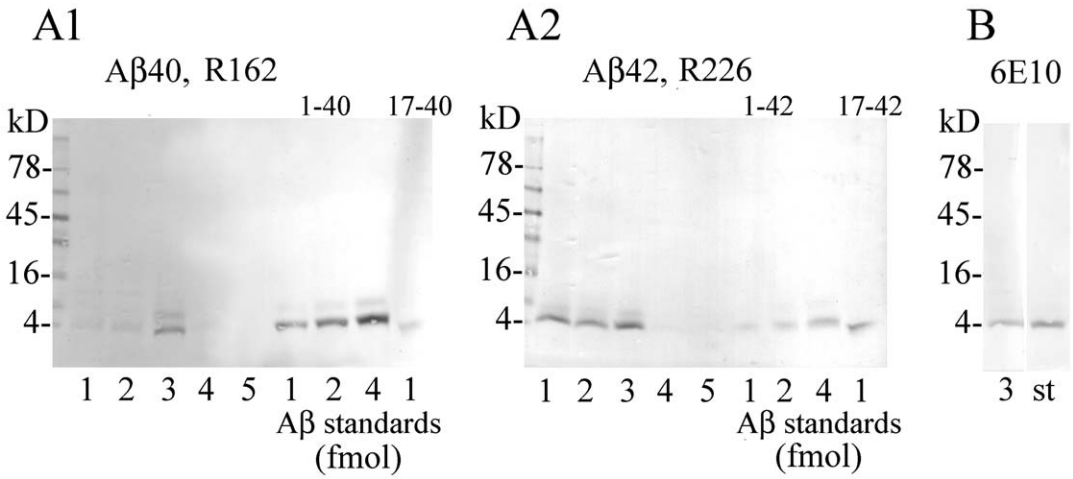


Figure 9. Properties of Aβ in plaque-rich cortex characterized by Western blotting. Panels A1 and A2 show Aβ40 and Aβ42 detected with pAbs R162 and R226, respectively, in blots of extracts (3 μg of total proteins per line) from cerebral cortex containing diffuse plaques of a 39-year-old subject with dup(15) (lane 1), of 51- and 52-year-old individuals with idiopathic autism (lanes 2 and 3), and of 48- and 47-year-old controls (lane 4 and 5). Blots reveal full-length Aβ, mainly Aβ42, in samples from plaque-positive subjects but not in controls. As standards, 1, 2 and 4 fmols of synthetic Aβ1–40, 17–40 (panel A1) and Aβ1–42, 17–42 (panel A2) were used. Panel B shows Aβ detected with mAb 6E10 specific for the N-terminal portion of Aβ in extract from the cortex of the 52-year-old subject (lane 3; 6 μg of protein per lane) and 4 fmol of synthetic Aβ1–40 (st). Panels A1, A2 and B demonstrate that in the extracts from diffuse plaque–positive cortical samples of autistic subjects, the levels of Aβ1–40 and 1–42 exceeded 1.5 fmol per 1 μg of extracted protein.
doi:10.1371/journal.pone.0035414.g009

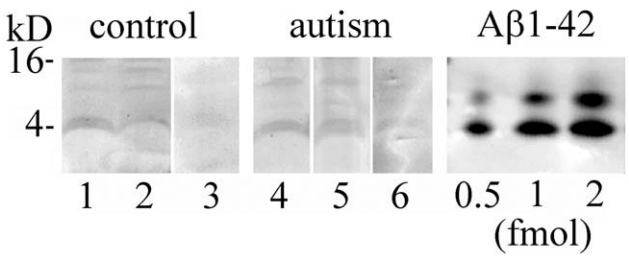


Figure 10. Detection by Western blots of Aβ in plaque-free subjects. Aβ42 detected with pAb R266 in lysates from cerebral cortex of control individuals 31, 32 and 51 years old (lanes 1–3, respectively), and individuals with idiopathic autism 8, 22, 29, and 29 years old (lane 4, 6, respectively). 40 μg of total lysate proteins were loaded per lane. Synthetic Aβ1–42 was used as a standard.
doi:10.1371/journal.pone.0035414.g010

guardians. The study was approved by the Institutional Review Boards for the New York State Institute for Basic Research in Developmental Disabilities; the University of California, Los Angeles; and Nemours Biomedical Research, duPont Hospital for Children, Wilmington. Clinical and genetic studies were performed as described previously [28]. Clinical characteristics were based on psychological, behavioral, neurological and psychiatric evaluation reports. To confirm a clinical diagnosis of autism, the Autism Diagnostic Interview-Revised (ADI-R) was administered to the donor family [79].

Molecular genetic evaluations, using antemortem peripheral blood samples and lymphoblast cell lines for eight of the dup(15) cases, included genotyping with 19–33 short tandem repeat polymorphisms from chromosome 15, Southern blot analysis of dosage with 5–12 probes, measurement of the methylation state at *SNRPN exon* α, as described [80], and array comparative genomic hybridization [81]. Duplication morphology was confirmed by fluorescent in situ hybridization [80].

In eight cases, tetrasomy, and in one case, hexasomy of the Prader-Willi/Angelman syndrome critical regions was detected. In eight cases, the origin of abnormality was maternal; in one case, the origin was not determined. In the examined dup(15) group, seven of nine subjects (78%) were diagnosed with autism or ASD, and seven had seizures. In six cases (67%), SUDEP was reported. In the idiopathic autism cohort, two subjects (8-year-old male, HSB4640, and 52-year-old male, BB1376), were diagnosed with the ASD (pervasive developmental disorder – not otherwise specified and high-functioning atypical autism, respectively). In all other cases, the clinical diagnosis of autism was confirmed with ADI-R.

One brain hemisphere was preserved for neuropathological and immunocytochemical studies. Methods and results of neuropathological evaluations of developmental abnormalities have been summarized in our previous reports [28,82]. The mean postmortem interval varied from 23.9 h in the dup(15) cohort to 19.6 h in the idiopathic autism cohort and 15.0 h in the control group. One brain hemisphere from each subject was fixed in 10% buffered formalin for a period ranging from six weeks to several months, dehydrated in a graded series of ethanol, infiltrated and embedded with PEG (Sigma) [83] and stored at 4°C. Tissue blocks were then cut into 50-μm-thick serial sections and stored in 70% ethyl alcohol. Two brains (AN17254 and BB1376) were embedded in celloidin, as described [82] and were cut alternatively into 200- and 50-μm-thick serial sections.

Immunocytochemistry and Confocal Microscopy

Brain Bank identification of the tissue samples is listed in Table 1, to maintain non-overlapping records of results of brains examined in different projects. Immunocytochemistry and confocal microscopy were applied to characterize (a) Aβ distribution in cells in the cerebral cortex, subcortical structures, cerebellum and brainstem and in diffuse plaques; (b) the Aβ peptide properties; and (c) Aβ distribution in endosomes, lysosomes, autophagic vacuoles, mitochondria and lipofuscin (Table 2).

mAbs 6E10 (Covance, Inc., Princeton, Inc.) and 6F3D (Novocastra Lab. Ltd., Newcastle, UK) were used to characterize the N-terminal portion of Aβ. mAb 6E10 recognizes an epitope in residues 4–13 of Aβ [84,85]. mAb 6F/3D recognizes an epitope in residues 8–17 of Aβ. The middle portion of Aβ was detected with mAb 4G8, which recognizes an epitope in residues 17–24 of Aβ [86]. The carboxyl terminus of Aβ was characterized with rabbit monoclonal antibodies Rabm38, Rabm40 and Rabm42, which detect $A\beta_{-38}$, $A\beta_{-40}$, and $A\beta_{-42}$, respectively [87]. The specificity of mAbs 4G8 and 6E10 for Aβ was verified in the examined postmortem human brain tissue by double immunolabeling with pAb R57 detecting APP C-terminal aa 671–695.

To detect intracellular Aβ peptides and amyloid in plaques, free-floating sections were treated with 70% formic acid for 20 minutes [88]. The endogenous peroxidases in the sections were blocked with 0.2% hydrogen peroxide in methanol. The sections were then treated with 10% fetal bovine serum in phosphate buffer solution (PBS) for 30 minutes to block nonspecific binding. The primary antibodies were diluted in 10% fetal bovine serum in PBS and sections were treated overnight at 4°C. The sections were washed and treated for 30 min with either biotinylated sheep anti-mouse IgG antibody or biotinylated donkey anti-rabbit IgG antibody diluted 1:200. The sections were treated with an extravidin peroxidase conjugate (1:200) for 1 h, and the product of reaction was visualized with diaminobenzidine (0.5 mg/mL with 1.5% hydrogen peroxide in PBS). After immunostaining, sections were lightly counterstained with cresyl violet. To detect fibrillar Aβ in plaques, sections were stained with Thioflavin S and examined in fluorescence.

Neurons with fibrillary tangles were immunolabelled with mAb Tau-1, detecting an epitope between amino acids 189 and 207 of the human tau protein sequence [89]. To detect abnormally phosphorylated tau with Tau-1, sections were pretreated with alkaline phosphatase (Sigma, Saint Louis, MO; Type VII-L, 400 μg/ml in PBS, pH 7.4, 0.01% H_20_2).

Double immunofluorescence for Aβ (mAb4G8) and for astrocytes (GFAP; rabbit polyclonal antibody, pAb, Sigma) was carried out to confirm the presence of Aβ in astrocytes. Confocal microscopy was applied to detect Aβ localized in neuronal cytoplasmic organelles. Aβ in lysosomes was detected by using lysosomal-associated membrane protein marker (LAMP1; Abgent, San Diego, CA). Early endosomes were immunodetected with rabbit pAb Rab5 (Ab13253; Abcam, Cambridge, MA), whereas autophagic vacuoles were immunolabelled with rabbit mAb LC3B (Cell Signaling Technology Inc., Danvers, MA). Mitochondria were detected with the rabbit mAb COXIV Alexa Fluor 488 conjugated (Cell Signaling Technology). To detect Aβ, brain sections were treated with 70% formic acid for 20 min, washed in PBS 2x 10 min and double- immunostained using mAb 4G8 and antibodies detecting markers of cytoplasmic organelles. Affinity-purified donkey antisera against mouse IgG labeled with Alexa Fluor 488 and against rabbit IgG labeled with Alexa Fluor 555 (both from Molecular Probes/Invitrogen) were used as secondary antibodies. TO-PRO-3-iodide (Molecular Probes/Invitrogen) was used to counterstain cell nuclei. Absence of cross-reaction was

Table 1. Material examined, cause of death, and the prevalence of epilepsy.

Group	Brain Bank number	Sex	Age (y)	Cause of death	Epilepsy. age of onset
dup(15)	AN14762	M	9	SUDEP	IE/10 m
dup(15)	AN06365	M	10	SUDEP	IE/8 m
dup(15)	AN09402	M	11	SUDEP	IE/10 m
dup(15)	AN07740	F	15	SUDEP	E/11 y
dup(15)	AN09470	F	15	Aspiration pneumonitis	–
dup(15)	AN03935	M	20	Cardiopulmonary arrest	–
dup(15)	AN05983	M	24	Pneumonia	IE/7 y
dup(15)	AN14829	F	26	SUDEP	E/16 y
dup(15)	AN11931	F	39	SUDEP	IE/9 y
Autism	AN03345	M	2	Asphyxia (drowning)	–
Autism	AN13872	F	5	Asphyxia (drowning)	–
Autism	AN08873	M	5	Asphyxia (drowning)	–
Autism	HSB4640	M	8	Asthma attack	E/8 y
Autism	AN01293	M	9	Heart failure	–
Autism	CAL105	M	11	Asphyxia (drowning)	E
Autism	IBR93-01	M	23	Seizure related	E/23 y
Autism	AN08166	M	28	Seizure-related	E
Autism	NP06-54	M	32	Brain tumor	–
Autism	AN17254	M	51	Heart failure	1 grand mal
Autism	BB1376	M	52	Heart failure	–
Control	UMB1706	F	8	Rejection of cardiac transplant	–
Control	UMB1670	M	14	Asphyxia (hanging)	–
Control	UMB4722	M	14	Multiple traumatic injuries	–
Control	BTB3960	F	25	Not known	–
Control	IBR291-00	M	32	Heart failure	–
Control	IBR212-98	F	33	Bronchopneumonia	–
Control	IBR38-98	F	43	Sepsis	–
Control	IBR457-96	M	47	Myocardial infarct	–

Sudden unexpected and unexplained death of subject with known epilepsy (SUDEP), Intractable epilepsy (IE), Epilepsy (E), Years (y), Months (m).
doi:10.1371/journal.pone.0035414.t001

confirmed as previously described [26]. Images were generated using a Nikon C1 confocal microscope system with EZC1 image analysis software.

Comparison of Intraneuronal Aβ Accumulation in Examined Cohorts

Semiquantitative estimation of intraneuronal Aβ was performed without knowledge of the subject's age, gender or clinical diagnosis or the neuropathological diagnosis of the tissue being analyzed. Evaluation was performed at a workstation consisting of Axiophot II light microscope, specimen stage with 3-axis computer-controlled stepping motor system (Ludl Electronics; Hawthorne, NY), CCD color video camera (CX9000 MicroBrightField Bioscience, Inc., Williston, VT) and stereology software (Stereo Investigator, MicroBrightField Bioscience Inc.). Grid size and the virtual test area were designated individually for each brain region to adjust to the region of interest size and shape. Intraneuronal Aβ accumulation has been estimated by four neuropathologists in 12 brain structures including frontal, temporal and occipital cortex, amygdala, thalamus, lateral geniculate body, sectors CA1 and CA4, and dentate gyrus in the hippocampal complex, Purkinje cells and dentate nucleus in cerebellum, and inferior olive in the brainstem. The number of 4G8-negative neurons and neurons with weak (<10 immunopositive granules per cell), strong (condensed mass of indistinguishable small and large immunoreactive granules) and medium (>weak and <strong) immunoreactivity was determined using a ×40 objective lens. For each subject, from 100 to 180 neurons were examined per region of interest in sections immunostained with mAb 4G8. Inspection of the entire cell cytoplasm by using micrometer screw contributed to precise rating of amyloid load in each examined neuron.

Differences in the estimated cytoplasmic neuronal Aβ load were examined using the Mann-Whitney U (Wilcoxon signed ranks) test or, for comparison of all three groups, the Kruskal-Wallis one-way ANOVA (an extension of the U test) [90]. Statistics were computed from pooled data from each group [dup(15) autism, idiopathic autism, control], where sampled neurons immunoreactivity was categorized as strong, medium, weak or none.

Western Blotting

Frozen temporal cortex samples from three control and three autistic subjects were homogenized in 10×volume of 10 mM TRIS buffer containing 0.65% NP-40, 1 mM EDTA and

Table 2. Antibodies used for immunocytochemistry, immunofluorescence and western blotting.

Name	Epitope or target	Dilution	Host	Application	Source
6E10	4–13 aa Aβ	1:10,000	M-m	ICH,CM, WB	Covance, Inc., Princeton, Inc. [84,85]
6F3D	8–17 aa Aβ	1:50	M-m	ICH	Novocastra Laboratories Ltd., Newcastle, UK
4G8	17–24 aa Aβ	1:8,000	M-m	ICH,CM, WB	IBR [86]
Rabm38	−38 aa Aβ	100 ng/mL	R-m	ICH	IBR
Rabm40	−40 aa Aβ	100 ng/mL	R-m	ICH	IBR [87]
Rabm42	−42 aa Aβ	100 ng/mL	R-m	ICH	IBR [87]
R57	APP C-terminal aa 671–695	1:3,000	R-p	CM	IBR
R226	36–42 aa Aβ		R-p	CM, WB	IBR
R162	Aβ C-terminus		R-p	CM, WB	IBR
LAMP 1	Lysosomes	1:400	R-p	CM	Abgent, San Diego, CA
Rab5 Ab13253	Early endosomes	1:100	R-p	CM	Abcam Inc., Cambridge, MA
LC3B	Autophagic vacuoles	1:100	R-m	CM	Cell Signaling Technology Inc., Danvers, MA
COXIV	Mitochondria	1:100	R-m	CM	Cell Signaling Technology Inc., Danvers, MA
GFAP	Astrocytes	1:400	R-p	CM	Sigma, Saint Louis, MO
AIF/IBA1 AF1039C	Microglia	1:200	G-p	CM	Abgent, San Diego, CA
Tau-1	Tau protein	1:1000	M-m	ICH	IBR

Mouse monoclonal (M-m), Rabbit monoclonal (R-m) or polyclonal (R-p), Goat polyclonal (G-p). Immunocytochemistry (ICH), Confocal microscopy (CM), Western blots (WB).

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Complete protease inhibitor coctail (Roche, Mannheim, Germany) in a Potter-Elvehjem homogenizer and sonicated for 2 minutes. Protein content in lysates was measured by BCA assay (Pierce). Forty μg of total lysate proteins were loaded per lane for PAGE in 8–15% gradient gels.

Tissue samples from formalin-fixed PEG or celloidin-embedded brains of three subjects with diffuse plaques detected by immunocytochemistry (39-year-old female diagnosed with dup(15) autism, a 51-year-old autistic subject, and a 52-year-old subject with atypical autism) and two subjects without plaques (48-year-old autistic and a 47-year-old control subject) were used for protein extraction. From 50-μm-thick sections, approximately 120 mm^2 of affected cortex was dissected (approximately 6 mm^3 of tissue), rehydrated in PBS and homogenized in Potter-Elvehjem homogenizer in PBS containing 0.5% sodium deoxycholate, 0.1% SDS and 1% NP-40 (RIPA buffer). After sonication two times for three minutes, the material was centrifuged at 16,000g for 20 minutes, and supernatants were collected as RIPA extracts. Protein content in the extracts was measured by the BCA assay (Thermo Scientific, Rockford, IL). For Aß detection with R162, R226, and mAb 6E10, the amounts of extracted proteins loaded per lane were 3, 3 and 6 μg, respectively. The proteins were subjected to PAGE in 8–15% gradient gels, transferred onto nitrocellulose and probed with antibodies specific for C-terminus of Aß40 (R162) and Aß42 (R226), and N-terminus–specific mAb 6E10.

Supporting Information

Figure S1 Neurons with low and high amyloid load. In control brains, the percentage of Aβ-positive neurons and their amyloid load is much lower in CA1 than in CA4 sector and is very low in the granule neurons in the dentate gyrus. The percentage of Aβ-positive neurons and amyloid load is significantly higher in the dup(15) autism cohort than in the control and idiopathic autism groups ($p<0.0001$), but the difference between idiopathic autism and control is insignificant. The characteristic feature of the LGB, inferior olive and dentate nucleus of control subjects is the very high percentage of Aβ-positive neurons and the highest amyloid load among the examined 12 structures. The increase of amyloid load is undetectable in the inferior olive and is minimal in the LGB and dentate nucleus of subjects with idiopathic autism and dup(15) autism.
(TIF)

Figure S2 Topography and morphology of neocortical diffuse plaques. Low magnification demonstrates diffuse plaques immunostained with mAb4G8 (17–24 aa) in frontal, temporal and occipital cortex (FC, TC and OC, respectively) in the brain of a 39-year-old female diagnosed with dup(15) autism, a 51-year-old autistic male, and a 52-year-old subject with atypical autism.
(TIF)

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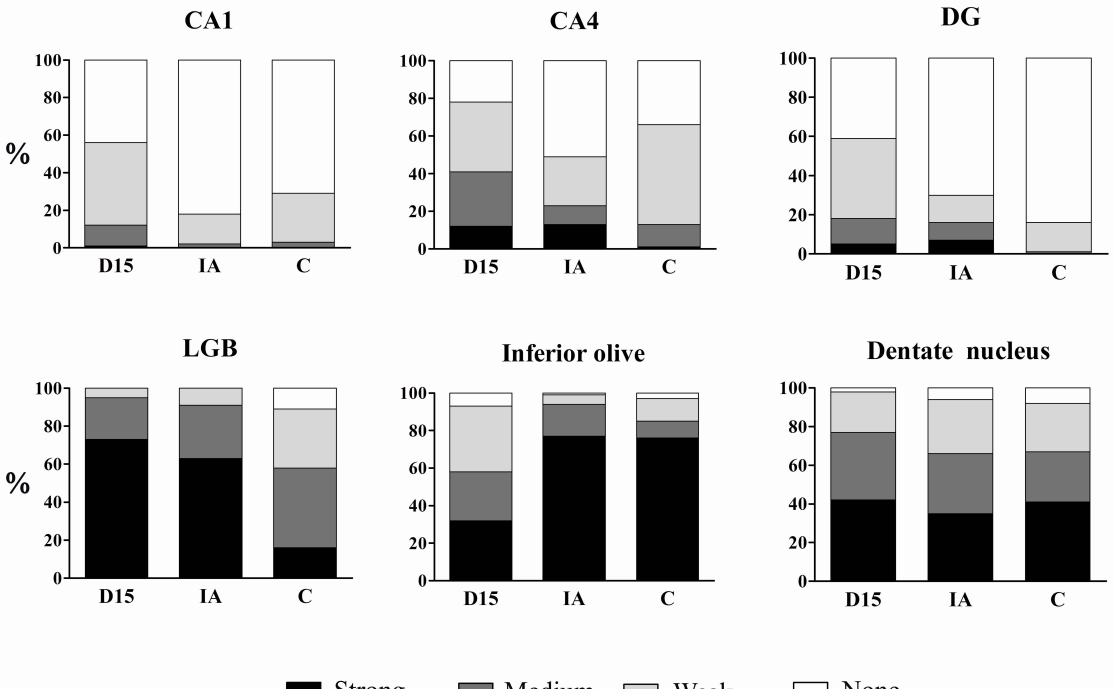
Author Contributions

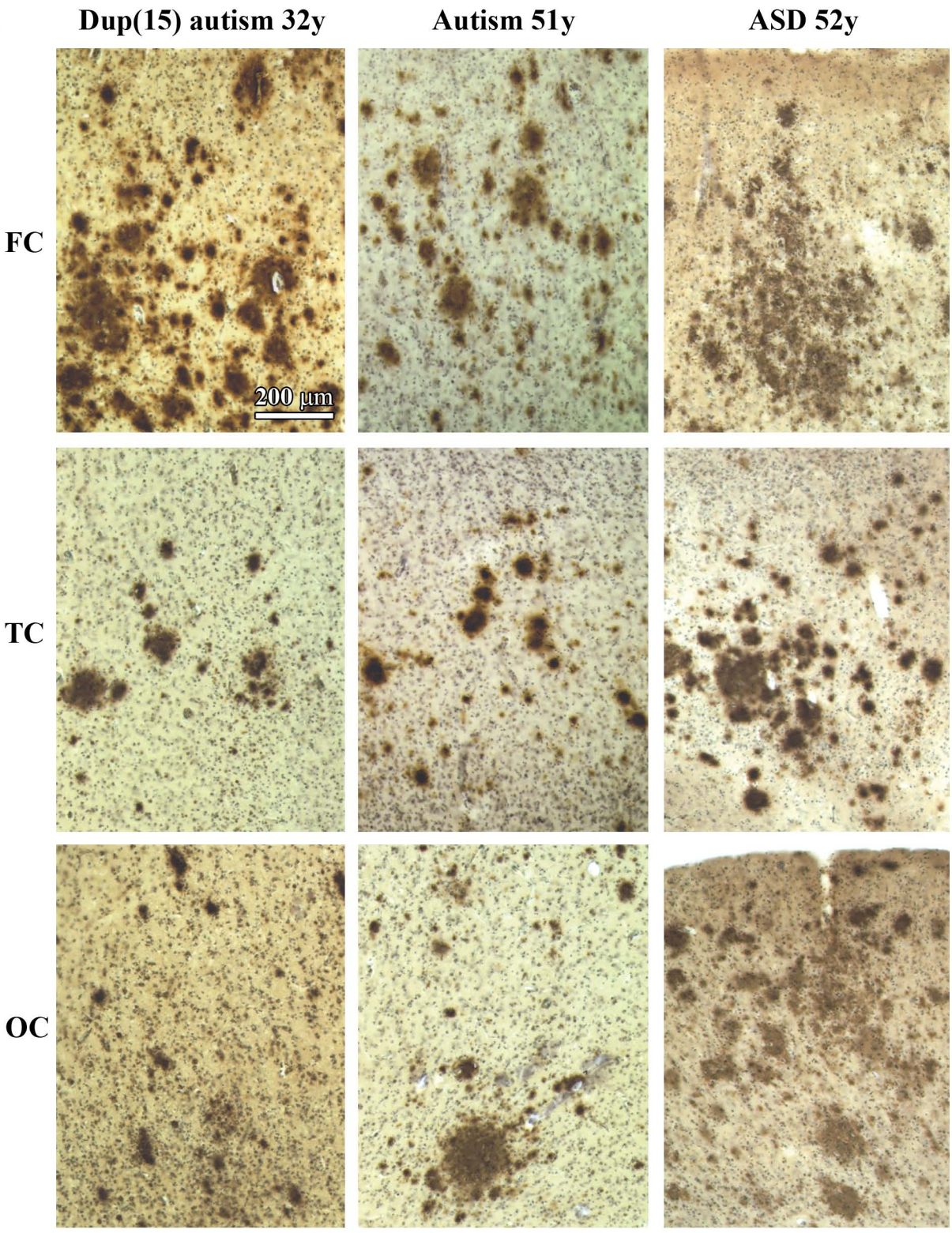
Conceived and designed the experiments: Jerzy Wegiel BR MJL. Performed the experiments: JF BMK Jarek Wegiel AC VC. Analyzed the data: KN HI SYM MF. Contributed reagents/materials/analysis tools: DLM PDM. Wrote the paper: Jerzy Wegiel NCS EHC WTB. Genetic diagnosis: NCS EHC MS. Clinical evaluation: NCS EHC ILC EL. Neuropathological evaluation: TW IK Jerzy Wegiel.

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CA1
CA4
DG
LGB
Inferior olive
Dentate nucleus
%
100
80
60
40
20
0
D15
IA
C
Strong
Medium
Weak
None


Dup(15) autism 32y
Autism 51y
ASD 52y
FC
TC
OC
200 µm

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Research Report

Contribution of olivofloccular circuitry developmental defects to atypical gaze in autism

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ABSTRACT

Individuals with autism demonstrate atypical gaze, impairments in smooth pursuit, altered movement perception and deficits in facial perception. The olivofloccular neuronal circuit is a major contributor to eye movement control. This study of the cerebellum in 12 autistic and 10 control subjects revealed dysplastic changes in the flocculus of eight autistic (67%) and two control (20%) subjects. Defects of the oculomotor system, including avoidance of eye contact and poor or no eye contact, were reported in 88% of autistic subjects with postmortem-detected floccular dysplasia. Focal disorganization of the flocculus cytoarchitecture with deficit, altered morphology, and spatial disorientation of Purkinje cells (PCs); deficit and abnormalities of granule, basket, stellate and unipolar brush cells; and structural defects and abnormal orientation of Bergmann glia are indicators of profound disruption of flocculus circuitry in a dysplastic area. The average volume of PCs was 26% less in the dysplastic region than in the unaffected region of the flocculus ($p<0.01$) in autistic subjects. Moreover, the average volume of PCs in the entire cerebellum was 25% less in the autistic subjects than in the control subjects ($p<0.001$). Findings from this study and a parallel study of the inferior olive (IO) suggest that focal floccular dysplasia combined with IO neurons and PC developmental defects may contribute to oculomotor system dysfunction and atypical gaze in autistic subjects.

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1. Introduction

Autism is a neurodevelopmental disorder characterized by deficits in social interactions and verbal and non-verbal communication, and restricted, repetitive behaviors (American Psychiatric Association, 2000). Among the most striking features of the social impairments in autism are deficits in coordinating visual attention with others. Making eye contact is one of the most fundamental aspects of human social behavior (Dawson et al., 2002). Face-to-face interactions and mutual gaze establish the first social relations and may be the driving force behind infants' interest in human faces long before the acquirement of vocal language (Gliga and Csibra, 2007; Hoehl et al., 2009). In triadic person-object-person interactions, eye gaze indicates another person's focus of attention and guides learning in the first months after birth (Striano and Reid, 2006). Numerous studies reveal abnormalities in processing information from the eyes (Johnson et al., 2005), with numerous implications for autistic subjects' social development (review by Hoehl et al., 2009). However, active avoidance of eye contact, poor eye contact or lack of eye contact reported very early in infancy appears to be the primary abnormality that is most likely caused by developmental defects within infant olivofloccular neuronal networks.

1.1. *Atypical gaze in autism*

Eye gaze-processing impairments appear early in the development of children with autism (Dawson et al., 1998; Mundy et al., 1986). Even high-functioning individuals with autism exhibit deficits in performing tasks involving mental interferences from viewing expression in the eyes (Baron-Cohen et al., 2001). Gaze-processing deficits result from impairment in using gaze to understand the intentions and mental states of other people (Baron-Cohen, 1995, 1999, 2001; Leekam et al., 1998, 2000). Children with autism demonstrate "atypical" gaze and frequently have stereotypies including eye-pressing and light-gazing. Several studies have revealed impairments in smooth visual pursuit in autism (Rosenhall et al., 1988; Scharre and Creedon, 1992; Takarae et al., 2004). Abnormalities of the oculomotor system, including atypical optokinetic nystagmus, gaze avoidance and stereotypic behaviors related to eyes, were reported in 91% of 34 children diagnosed with autism (Scharre and Creedon, 1992). A study of 60 high-functioning individuals with autism revealed pursuit eye movement deficits (Takarae et al., 2004). The subjects had a reduced closed-loop pursuit gain when tracking both oscillating and ramp targets. More apparent deficits after mid-adolescence suggest reduced maturational development of the pursuit system in autism. Bilateral disturbances in the ability to use internally generated extraretinal signals for closed-loop pursuit suggest defects in the frontostriatal or cerebellar circuitry. An fMRI study using saccadic and pursuit eye movement paradigms revealed reduced activation in cortical eye fields and cerebellar hemispheres in autistic subjects (Takarae et al., 2007).

1.2. *The role of flocculus in eye movement control*

Experimental studies indicate that the cerebellar flocculus is the part of the oculomotor system involved in both the olivo-cerebellar circuit and the vestibulooccular reflex arc. The flocculus exerts a specific inhibitory modulation of both excitatory and inhibitory branches of the vestibulo-ocular reflex pathways to the extraocular muscles. This modulation is mediated through inhibitory projections of floccular Purkinje cells (PCs) to the vestibular nuclei neurons, described as flocculus target neurons (reviewed by Du Lac et al., 1995; Sato and Kawasaki, 1991). The flocculus is also a region of vestibular afferent signal convergence, through the input of mossy fibers from the vestibular nuclei, and of visual signals received via the climbing fibers of the inferior olive (IO) (Sato and Kawasaki, 1991).

A substantial number of PCs in the flocculus of the monkey receive converging visual inputs from functionally distinct portions of the retina and are involved in oculomotor control during slow eye movements. The flocculus provides the oculomotor system with eye position information during fixation and with velocity information during smooth pursuit and participates in the control of oculomotor functions (Noda and Suzuki, 1979; Zee et al., 1981). Velocity plays a stronger role (64%) than position (36%) in determining firing rate modulation during circular pursuit (Leung et al., 2000). The majority of visual PCs (85%) are identified as horizontal gaze-velocity neurons. The presence of position and velocity signals in the flocculus and paraflocculus suggests that these two cerebellar regions generate the position and velocity signal that are used to control eye motion (Leung et al., 2000). Moreover, PCs in the flocculus help to coordinate eye and head movements during active gaze shifts by modulating the vestibulo-ocular reflex (Belton and McCrea, 1999).

1.3. *Olivo-cerebellar system*

From a movement perspective, the olivo-cerebellar system has been considered to be involved in motor learning (Hesslow and Yeo, 2002; Highstein et al., 2005; Ito, 2001) and in the timing of motor execution (Lang et al., 1999). The olivo-cerebellar system is involved in both somatomotor and oculomotor cerebellar control. Electrophysiological studies have revealed that neurons in the dorsal cap of Kooy and the adjacent ventrolateral outgrowth of the IO control eye movements via their climbing fiber projections to the cerebellar flocculus, whereas neurons in the principle olive are involved in control of limb and digit movements via their climbing fiber projections to the lateral part of the cerebellar hemisphere (reviewed by Ito, 1984 and Voogd and Bigaré, 1980).

Neuropathological studies indicate that the oculomotor circuit is often developmentally affected in individuals with autism. Our study of serial sections of the brain revealed multiregional dysregulation of cerebellum development in nine of 13 (69%) subjects with autism. The presence of cerebellar floculonodular dysplasia in six autistic subjects (46%), focal dysplasia in the vermis of one subject, heterotopia in one case and focal cerebellar hypoplasia in one subject reflected a high susceptibility of the cerebellum to developmental defects (Wegiel et al., 2010). The presence of olivary dysplasia in three of five subjects with autism and of ectopic neurons related to the olivary complex in two cases reported by Bailey et al. (1998) suggests that both components

of the oculomotor circuitry are prone to developmental defects.

The aim of this study was to detect defects of the olivo-cerebellar circuitry that may be responsible for the altered oculomotor activity, atypical gaze, impairments in smooth pursuit, altered movement perception and deficits in facial perception observed in autism. The main target of this study is the flocculus, known to be involved in oculomotor circuitry.

2. Results

Postmortem study of the brain of 12 autistic subjects and 10 control subjects revealed dysplasia of the flocculus (Fig. 1a and b) in eight autistic (67%) and two control (20%) subjects (Table 1). Clinical records revealed poor, very poor or no eye contact in seven of the eight subjects (88%) with floccular dysplasia (Table 2). Clinical records also revealed difficulties in establishing and maintaining eye contact in two autistic subjects without signs of floccular dysplasia in the examined cerebellar sections; however, histopathological evaluation was limited because of the loss of part of the flocculus during brain removal. In one case with floccular dysplasia, clinical records were not available. The other factor limiting correlations between floccular pathology and eye contact deficiency is examination of only one cerebellar hemisphere. The partial loss of flocculus tissue noted in three other cases with floccular dysplasia results in downgrading of the percentage of dysplastic area. Therefore, only the presence of floccular dysplasia in eight cases (seven with clinical records of eye contact deficit, and one without records) is unbiased. The limited clinical records of the control subjects do not contain any reference to altered behavior or eye contact deficit. Therefore, the impact on eye contact of the very small and moderate-size floccular dysplasia observed in the two control subjects cannot be determined. Dysplasia was also found in the nodulus, but abnormalities were equally common in subjects with autism (9/12; 75%) and controls (8/10; 80%), and the affected area was smaller than in the flocculus (Fig. 1c).

2.1. *Quantitative characteristics of the flocculus and PCs*

The average volume of the flocculus was greater in the subjects with autism (226 mm^3) than in the controls (179 mm^3; $p<0.002$) (Table 1). The average volume of the dysplastic area in eight subjects with autism was greater (7.2 mm^3) than in two controls (4.4 mm^3). Significant inter-individual differences in the volume of the dysplastic area were observed among eight subjects with autism (from 0.2 to 17.3 mm^3) and in two controls (1.6 and 7.3 mm^3). Dysplastic changes modified proportions between the major flocculus cytoarchitectonic subdivisions. There was a much smaller contribution of the molecular layer (29%) in the dysplastic than in the unaffected flocculus (40%), but a larger-volume contribution of the granule cell layer (58%) in the dysplastic flocculus than in the unaffected area (46%). However, contributions of the white matter were almost the same in the dysplastic (13%) and the unaffected (14%) area.

Application of the Nucleator revealed that the volume of the PCs in eight subjects with autism was reduced in the dysplastic area by 24% (6240 μm^3) in comparison to the non-dysplastic area (8230 μm^3) ($p<0.01$). In two control subjects with dysplastic changes, the difference between the volume of PCs in the dysplastic (7477 μm^3) and the non-dysplastic (9367 μm^3) areas was 20% ($p<0.01$). A broader pattern of cerebellar developmental alterations is reflected in the 26% smaller volume of the PCs' soma in the entire cerebellum in seven subjects with autism (8382 μm^3) than in seven control subjects (11,268 μm^3) ($p<0.001$) (celloidin protocol only).

2.2. *Topography and morphology of floccular dysplasia in autism*

Dysplastic changes were observed in the most rostral portion of the flocculus (Fig. 1a and b). There was profound distortion

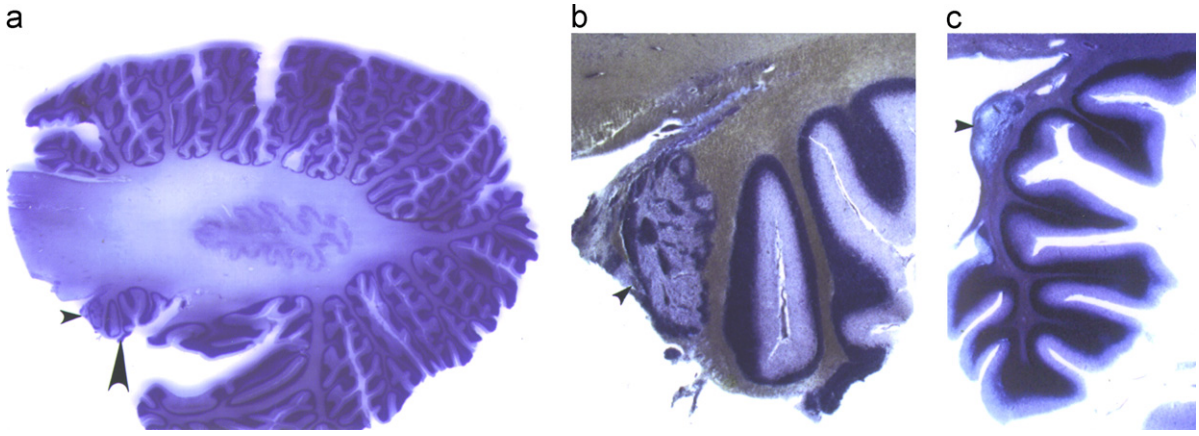


Fig. 1 – Topography of dysplastic changes in the flocculus and nodulus. Dysplastic changes in the rostral portion of the flocculus of a 36-year-old subject with autism (a, b). Low magnification (a) illustrates the proportions between the size of the cerebellum, flocculus (large arrowhead) and dysplastic area (small arrowhead). (b) shows the loss of spatial organization of the granule and molecular layers in the dysplastic portion (small arrowhead) of the flocculus. (c) illustrates the topography and morphology of dysplastic changes (small arrowhead) in the rostral portion of the nodulus of a 7-year-old subject with autism. 200-μm-thick cresyl violet-stained celloidin sections.

Table 1 – The volume of the flocculus dysplastic area.

No.	Group	Case/brain number	Flocculus volume (mm^3)		Dysplasia: % of total flocculus volume
			Total	Dysplastic area	
1	A	IBR-425-02	221	0.2	0.1
2	A	AN13961	225	11.5	5.1
3	A	HSB-4640	217	3.6	1.7
4	A	AN01293	174	0.0	0.0
5	A	B-5223	287	2.3	0.8
6	A	AN09730	240	4.2	1.7
7	A	AN08166	303	0.0	0.0
8	A	NP06-54	179	0.0	0.0
9	A	AN16961	228	17.3	7.6
10	A	AN08105	270	12.1	4.5
11	A	AN09714	170	0.0	0.0
12	A	AN12698	198	6.5	3.3
		Average	226	7.2	3.1
		SE	13	2.1	0.9
1	C	AN2456	177	0.0	0.0
2	C	UMB-4898	148	0.0	0.0
3	C	UMB-1708	224	0.0	0.0
4	C	UMB-1670	176	0.0	0.0
5	C	UMB-4722	155	7.3	4.7
6	C	UMB-1846	154	0.0	0.0
7	C	UMB-4543	170	0.0	0.0
8	C	UMB-1576	196	1.6	0.8
9	C	IBR-291-00	187	0.0	0.0
10	C	AN11184	203	0.0	0.0
		Average	179	4.4	2.7
		SE	8	2.9	2.0
		$P <$	0.002		

The average dysplastic region volume and percentage were calculated only for cases with dysplasia.

of the granule, molecular and PC layers in the dysplastic portion of the flocculus. Granule cells did not form a layer, but instead, islands and a labyrinth of thin, irregular-shaped cellular septa. The granule layer was mixed with an altered molecular layer and small islands of white matter. PCs were sparse and were dispersed in an abnormal granule cell and molecular layer, and in the white matter. Dysplastic changes were also detected in the most rostral portion of the nodulus, but the size of dysplasia was smaller than in the flocculus (Fig. 1c).

In the unaffected portion of the flocculus, immunocytochemistry using anti-calbindin mouse mAb D-28 revealed that the PCs' dendritic tree (Fig. 2a) was the major factor determining the volume and structure of the molecular layer. Parallel bundles of PCs' axons are an integral component of the cytoarchitecture of granule cell layer and white matter (Fig. 2b). The dysplastic area was characterized by the loss of cortical lamination, and the loss of neurons' spatial orientation and their morphological characteristics limiting or making impossible subclassification of such large neurons as Purkinje cells, and Golgi and Lugaro neurons (Fig. 2c–f). Striking deficits in PCs in dysplastic flocculus coexist with defects of axons and deficits of dendritic arborization (Fig. 2e).

Immunostaining with anti-calbindin (Fig. 2) and especially with anti-parvalbumin antibodies (Fig. 3) revealed not only severe dendritic abnormalities but also severe alterations of the PC axons in the dysplastic areas. Almost all axons showed alternative thickening and thinning, giving the axons a bead-like morphology. Moreover, immunostaining with anti-parvalbumin antibody revealed almost a complete lack of basket and stellate neurons in the developmentally distorted molecular layer in the dysplastic flocculus (Fig. 3b).

In control subjects, the study of distribution of calretininn-positive unipolar brush cells (UBCs) revealed a typically low number of UBCs in all lobules except the flocculus, with an increased number of UBCs (Fig. 4a–d). However, in the dysplastic area of the flocculus of autistic subjects, the number of calretinin-positive UBCs was reduced. In many islands of loosely arranged granule cells, UBCs were absent. The presence of calretinin-positive fibers several times thicker than normal suggests developmental abnormalities or dystrophic alterations (Fig. 4f).

Severe alterations of the number, distribution and morphology of all types of neurons in the dysplastic portion of the flocculus were associated with total loss of typical vertical Bergmann glia fibers in the molecular layer (Fig. 5a and b). GFAP-positive cells were dispersed within the developmentally modified molecular layer, but the lack of typical topography, orientation and morphology of GFAP-positive glia makes impossible the differentiation of Bergman glia and cortical astrocytes. Another sign of dysplastic flocculus was abnormally large clusters of GFAP-positive glia (Fig. 5c).

In the dysplasia-free portions of the flocculus, a few serotonin transporter (ST51)–immunopositive axons traverse

Table 2 – Psychiatric and neurological evaluation of autistic subjects including eye contact.

Case/ brain number	Psychiatric and neurological evaluation	Eye contact
IBR-425-02	Regression at age of 2 years. Tantrums. Self-injurious behavior when upset. Hyperactivity. Asthma	In general, poor eye contact, but on occasion, a brief eye contact with other people. (Flocc.D.: 0.1%)
AN13961	Hypotonia. Seizures (age of onset, 14 m)	No eye contact. (Flocc.D.: 5.1%)
HSB-4640	Regression at age of 18 m Asthma from age of 2.5 years to death	Gaze directed at the objects of interest but not at people. (Flocc.D.: 1.7%)
AN01293	Moderate intellectual deficit	No records. (Flocc.D.: 0.0%)
AN11206	Severe autism. Intellectual deficit. Temper tantrums. Self-injurious behavior. Hyperactivity. Sleep disorder. Hypotonia. Abnormal gait. Allergy	No eye contact at age of 1.5 to 4 years. Poor eye contact at age from 4 to 15 years. (Flocc.D.: 0.8%)
AN09730	Tourette's syndrome, obsessive-compulsive disorder, mania. Self-injurious behavior with head-banging and tantrums. Seizures (age of onset, 15 y)	No eye contact during childhood. As adult, patient looked at people, as looking at his reflection in people's eyes. (Flocc.D.: 1.7%)
AN08166	High-functioning autism. Pervasive developmental disorder. Intermittent explosive personality disorder. Rigidity, obsessiveness. Hypotonia. Tremor Seizures (age of onset, 22 y)	Difficulty establishing and maintaining eye contact. Poor pursuit and limitation of upgaze. (Flocc.D.: 0.0%)
NP06-54	No records	No records. (Flocc.D.: 0.0%)
AN16961	Functional regression at age of 16 month. Obsessive-compulsive disorder. Anxiety. Aggression. Severe self-injurious behavior. Severe intellectual deficit. Allergy	Lack of "typical" or reciprocal eye gaze, but in adulthood sporadically a brief eye contact was noticed. (Flocc.D.: 7.6%)
AN08105	Recurrent depression. Anxiety, irritability, aggression, self-injurious behavior. Hyperactivity. Moderate intellectual deficit (IQ 48). Allergy, asthma. Hypothyroidism	Very poor eye contact (avoiding eye contact and difficulty looking people in the face). Strabismus. (Flocc.D.: 4.5%)
AN09714	Intellectual deficit. Abnormal gait (walking like "drunk"), might be related to severe hypoplasia of cerebellar lobes 1–4 affecting approximately 25% of cerebellar cortex. (Case B-7090; Wegiel et al., 2010) Seizures (age of onset, 3 y)	The only record indicates that at early infancy, child was unable to make eye contact. However, there is no record of eye contact deficit in childhood or adulthood. (Flocc.D.: 0.0%)
AN12698	Mild intellectual deficit. Abnormal gait	Clinical documentation not available. (Flocc.D.: 3.3%)

Flocc. D., the percent of the flocculus volume occupied with dysplasia.

the granule cells and molecular layer (Fig. 6a). In contrast to the moderate serotonergic innervation in the unaffected part of the flocculus, the dysplastic area was characterized by a several-fold increase in the number of ST-51-positive fibers. The largest serotonergic bundles of fibers were observed on the periphery of the dysplastic areas, frequently mixed with loosely arranged cells with the morphology of granule cells (Fig. 6b). The characteristic feature of the disproportionally dense network of serotonergic fibers within the dysplastic, loosely arranged granule cell layer was a large number of varicosities (Fig. 6c).

3. Discussion

3.1. *Cerebellar pathology in autism*

Neuroimaging and neuropathological studies indicate that cerebellar abnormalities are one of the most consistent findings in autism (Bauman and Kemper, 1996; Courchesne et al., 2001; Kemper and Bauman, 1993; Ritvo et al., 1986; Whitney et al., 2008, 2009). MRI studies have shown smaller vermian lobules VI andVII, which are likely due to neuronal hypoplasia (Bauman and Kemper, 1996; Courchesne et al., 1989, 2001; Hashimoto et al., 1995; Kaufmann et al., 2003). Neuropathological studies revealed that the cerebellum is frequently affected by such developmental abnormalities as defects of migration with heterotopias, focal nodulus and flocculus dysplasia, other forms of vermis and cerebellar folia hypoplasia, and sporadically by focal polymicrogyria (Wegiel et al., 2010, 2012). A review by Palmen et al. (2004) noted a reduced number of PCs in 72% of the reported autism cases. Both qualitative (Kemper and Bauman, 1993) and quantitative studies (Bailey et al., 1998; Fatemi et al., 2002) revealed a regional decrease in the number of PCs and the prenatal loss of PCs (Whitney et al., 2008, 2009). The reduction by 24% in PC size in adult autistic subjects compared to the age-matched control subjects was considered by Fatemi et al. (2002) a marker of PC atrophy. Reduction in the volume of PCs by 31% (in comparison to age matched control), of the dentate nucleus by 25% and of the IO by 14% in 4- to 8-year-old autistic subjects ($p<0.001$) demonstrates that different components of brainstem-cerebellar circuits are affected by developmental defects and that the range of deficits is brain

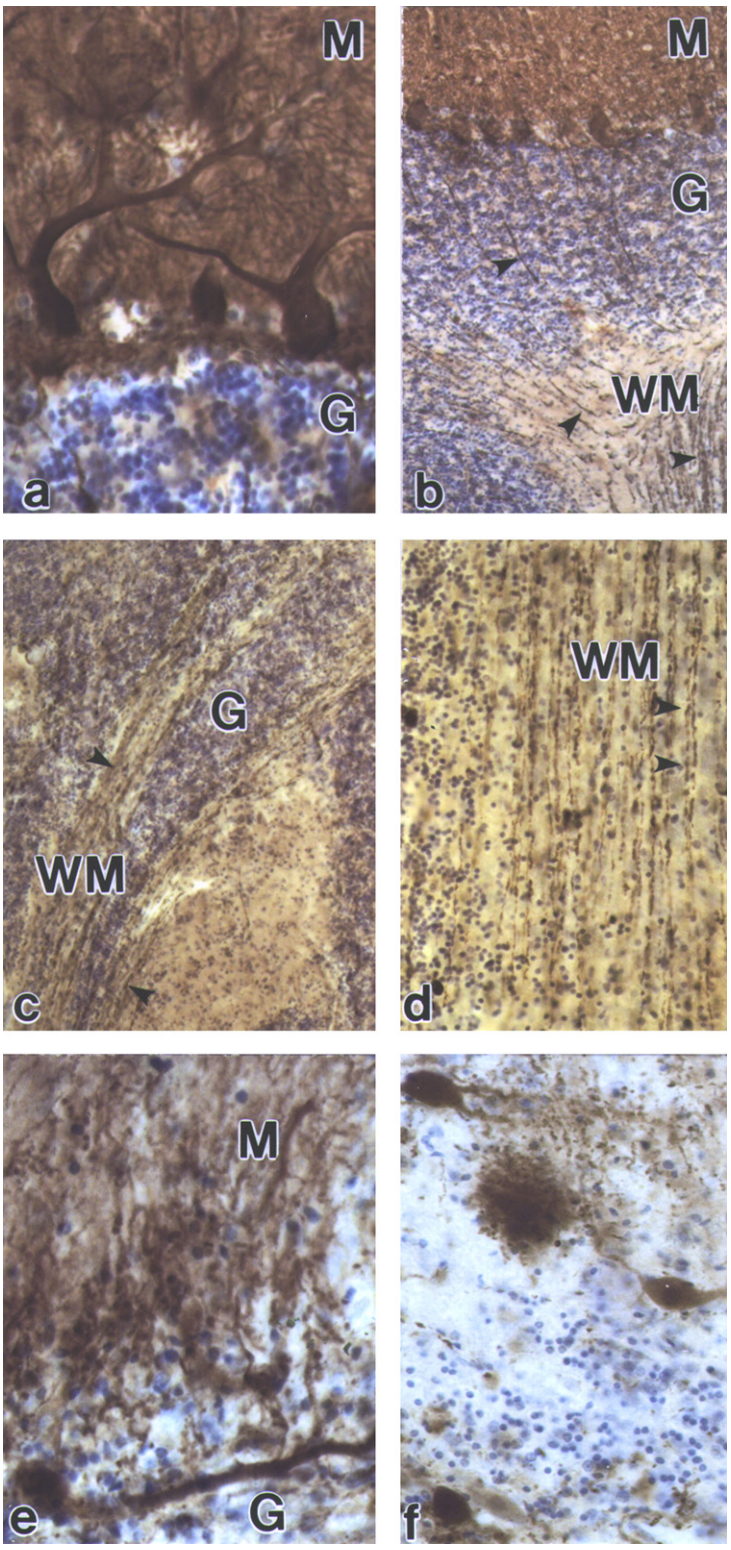


Fig. 2 – Calbindin immunostaining. Non-dysplastic (a, b) and dysplastic (c–f) portions of the flocculus of an autistic subject immunostained with anti-calbindin D-28k mouse mAb. (a) illustrates the morphology of PCs and their dendritic tree in the molecular (M) layer in the non-dysplastic portion of the flocculus, whereas low magnification (b) shows the morphology of the PCs' axons (arrowheads) penetrating the granule (G) layer and white matter (WM). In the dysplastic part of the flocculus of the same subject (c, d), irregular islands of loosely arranged granule (G) neurons are mixed with white matter-like areas (WM) accentuated by bundles of calbindin-positive PCs axons (small arrowheads). Almost all PCs display abnormal morphology of axons, with an alternative increase and reduction of the axon diameter (bead-like axons) (d). (e) demonstrates dendritic arborization defects of calbindin-positive PCs. (f) shows the fuzzy border of the island of granule neurons with calbindin-positive, poorly differentiated large and moderate-size neurons. This dysplastic area illustrates the profound defects of cortex cytoarchitecture, with the loss of neurons' spatial orientation, structure and connectivity.

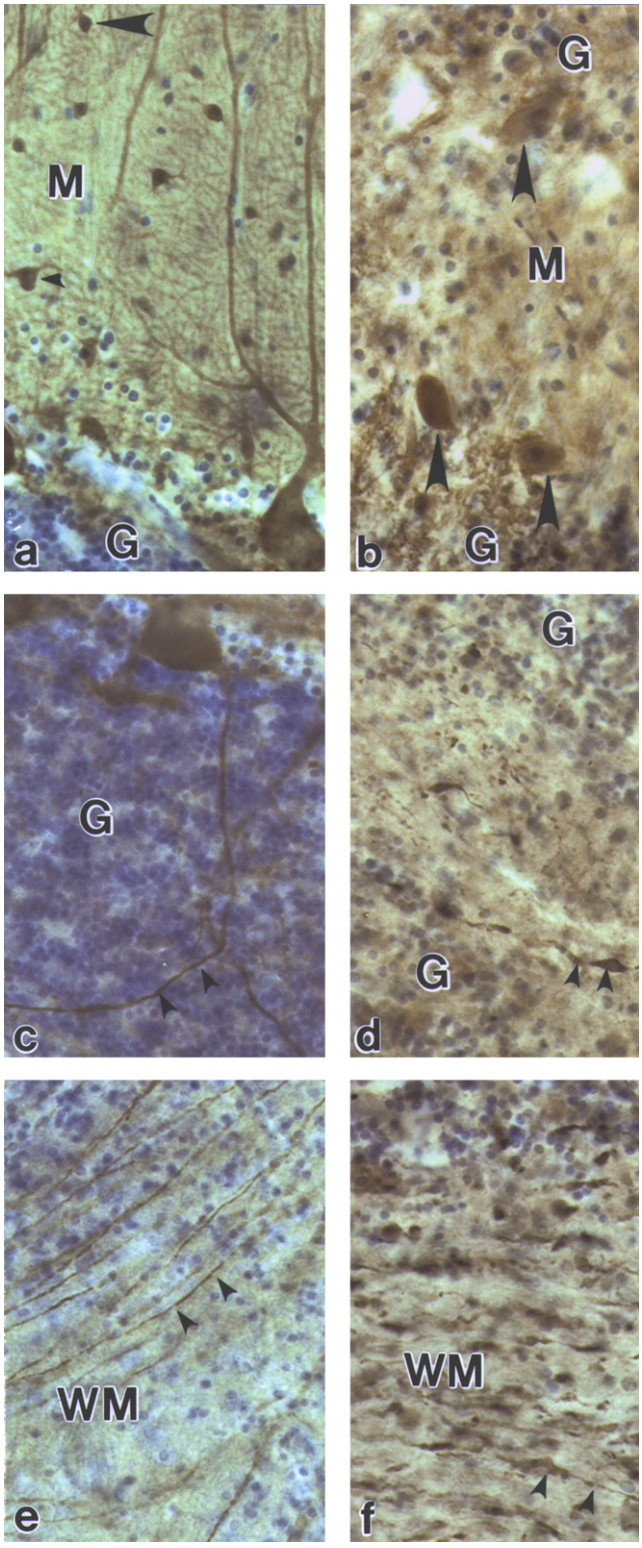


Fig. 3 – Parvalbumin immunostaining. Non-affected (a, c, e) and dysplastic (b, d, f) parts of the flocculus of an autistic subject immunostained with rabbit pAb. (a) shows the soma and dendritic tree of PCs, basket cells (small arrowhead) in the internal one-third of the molecular layer, and stellate cells (large arrowhead) in the external two-thirds of the molecular layer in the non-affected portion of the flocculus. (b) illustrates the dysplastic area with a few large, parvalbumin-positive neurons (large arrowheads) located in the fuzzy border zone between poorly defined granule (G) cells islands and abnormal molecular (M) layer devoid of PCs' dendrites, stellate and basket neurons but enriched in ectopic granule cells. (c) and (e) show the non-affected part of the flocculus with normal axons of PCs (pairs of small arrowheads) in the granule (G) cells layer and in the subcortical white matter. (d) and (f) demonstrate axonal abnormalities with alternative thick and thin segments (bead-like axons) (pairs of arrowheads) within dysplastic granule (G) cell islands and white matter (WM).

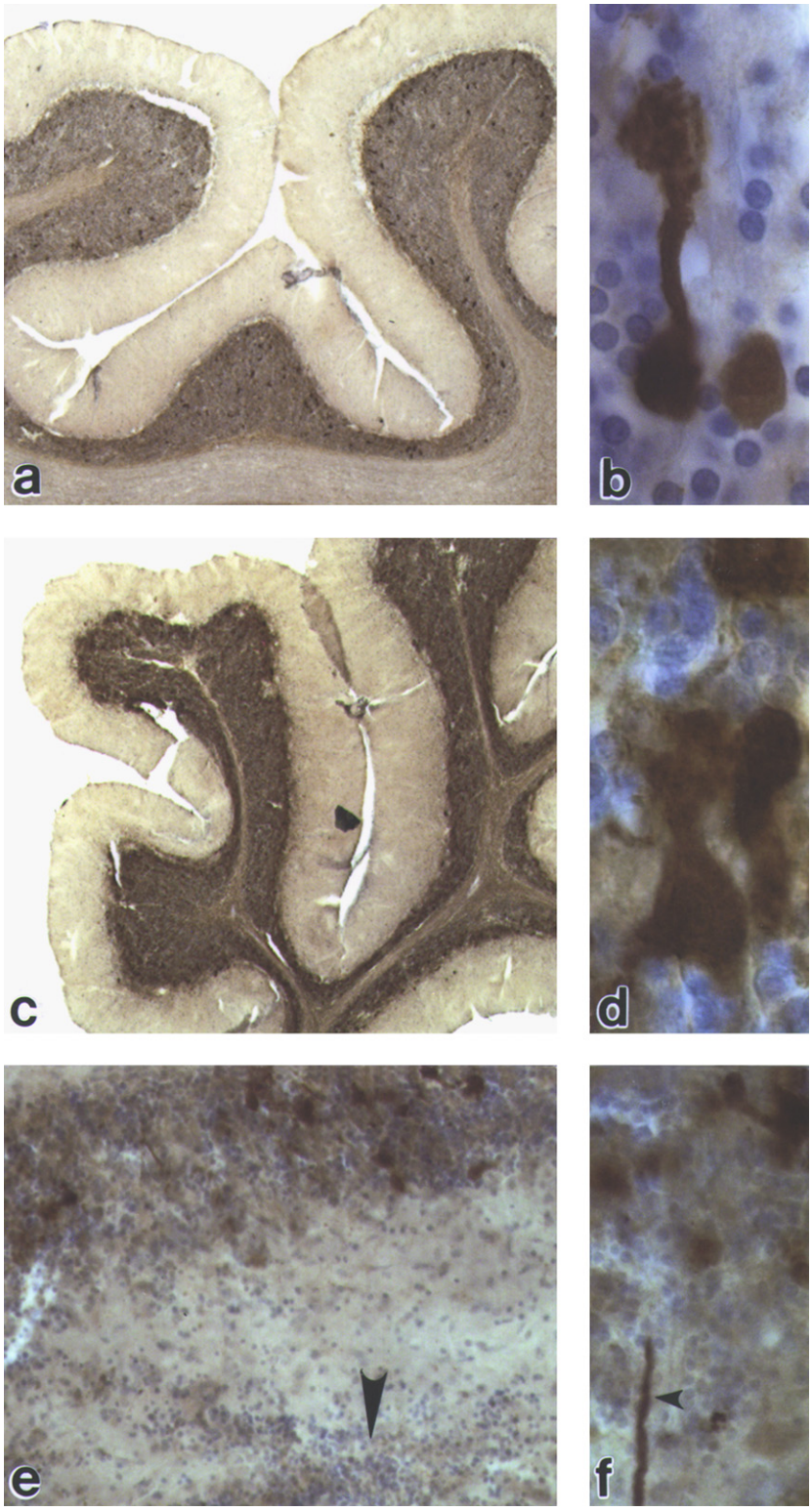


Fig. 4 – Immunostaining of unipolar brush cells (UBCs). Calretinin immunostaining in lobule II (a, b), not affected (c, d) and dysplastic (e, f) parts of the flocculus in an autistic subject. (a) shows the typical low number of UBCs in the granule cell layer in lobule II. (c) demonstrates the typical high number of UBCs in the granule cell layer in the non-affected part of the flocculus. High magnification shows calretinin-positive UBCs with a single dendritic process terminating with a compact arrangement of short brush-like dendrioles in lobule II (b) and clusters of UBCs in the non-dysplastic part of the flocculus (d). Anti-calretinin immunostaining demonstrates reduction and, in some areas, absence of UBCs (large arrowhead; e) as well as striking thickening of calretinin-positive fibers (small arrowhead; f) in dysplastic flocculus.

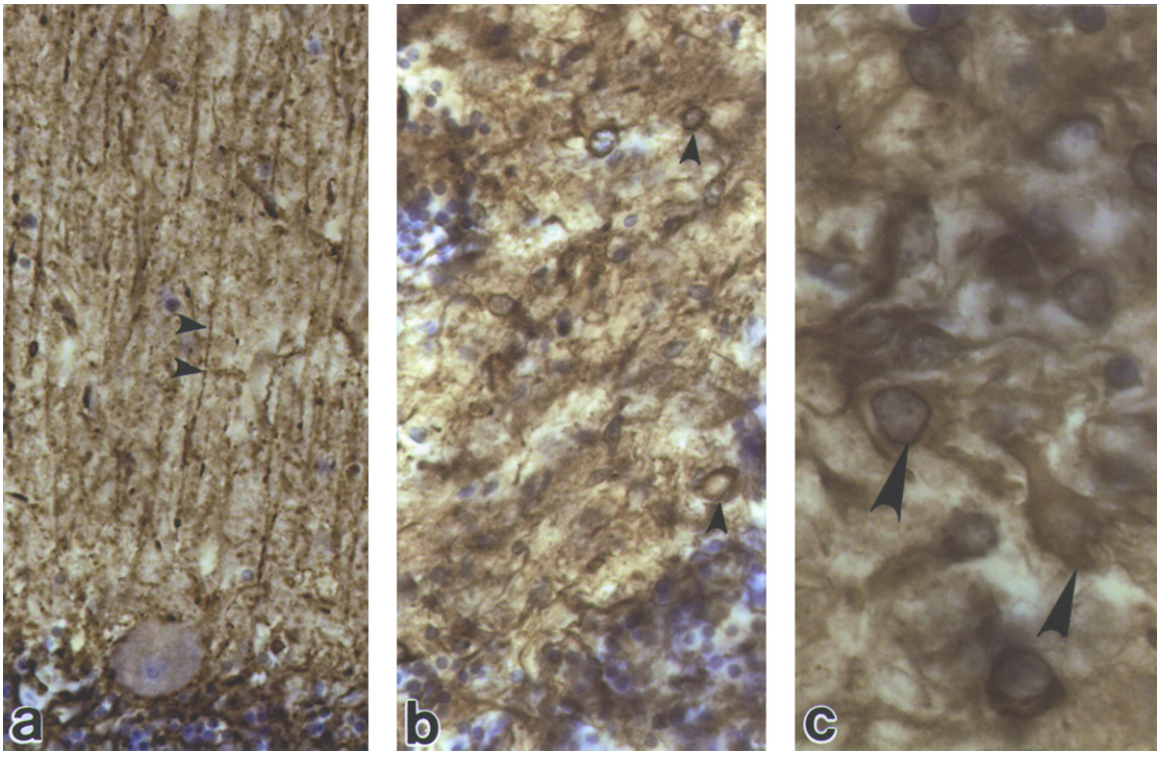


Fig. 5 – GFAP in Bergmann glia and astrocytes. Anti-GFAP immunostaining reveals numerous straight Bergmann fibers (arrowheads) in the molecular layer in the control subject (a), whereas dysplastic flocculus of an autistic subject is characterized by the total loss of the regular arrangement of Bergmann cell bodies in the borderline between the granule cell and molecular layer, and loss of parallel Bergmann fibers within the molecular layer. GFAP-positive glial cells (arrowheads) are dispersed within the molecular layer (b) or form clusters (c), but because of morphological alterations, distinguishing Bergmann glia from cortical astrocytes is impossible.

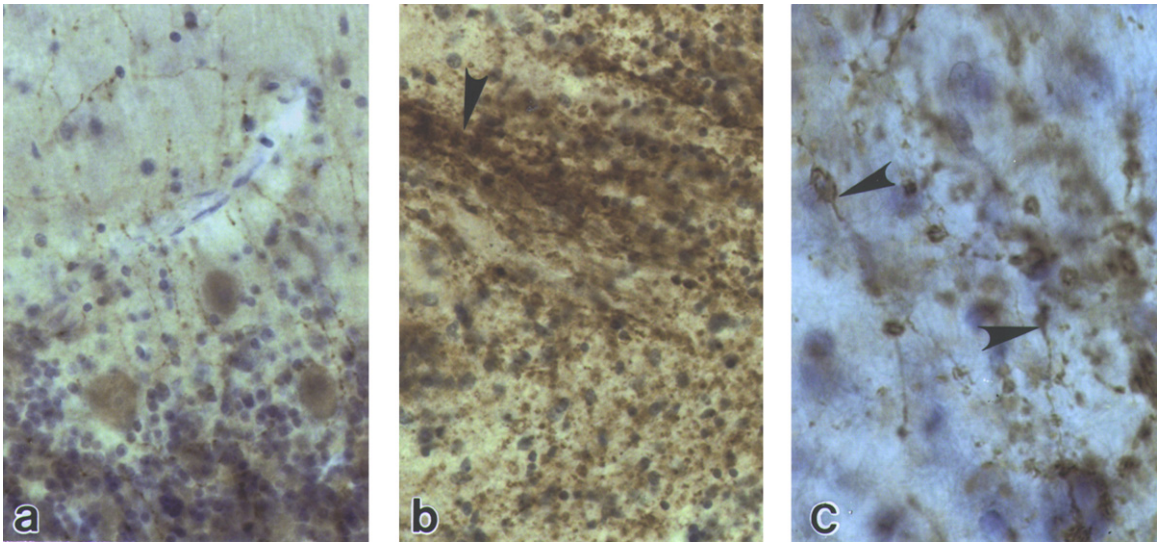


Fig. 6 – Serotonin transporter. In an autistic subject, immunostaining with ST51 revealed a moderate amount of serotonin transporter-positive fibers in the molecular and granule cell layers in the non-dysplastic part of the flocculus (a). In dysplastic regions, granule cell islands are penetrated with numerous dense bundles of serotonergic fibers (arrowhead; b). Dysplastic islands with very low numerical density of granule cells reveal disproportional massive serotonergic innervation and very numerous varicosities of ST-51-positive axons (c).

region-specific (Wegiel et al., submitted for publication). This broad spectrum of structural changes appears to define a high vulnerability of the cerebellum to developmental alterations, which may contribute to the autism phenotype. As reported here, the dysplastic changes in the flocculus appear to be a peculiar form of cerebellar focal developmental

defect, resulting in profound local alterations of the cytoarchitecture and a disorganization of the neuronal circuits involved in eye movements.

3.2. The role of altered Bergmann glia in spatial disorganization in the dysplastic flocculus

The profound spatial disorganization of the granule cell, molecular and PC layer in the dysplastic part of the flocculus indicates that the mechanisms controlling cortical folding, neuronal migration, spatial arrangement and connectivity during flocculus development are altered in the rostral flocculus portion of the majority of individuals with autism. Neuronal migration in different domains of the cerebellar cortex is regulated by domain-specific factors. Granule cell migration from the external granular layer to the internal granule layer is guided by surface-mediated interactions with Bergmann glial fibers, which traverse the developing molecular layer (Rakic, 1971, 1981). Migration to a final destination within the granule layer is modulated in response to local environmental cues (Komuro and Rakic, 1998). The lack of parallel Bergmann fibers and the spatial disorientation of their processes in the molecular layer of the dysplastic flocculus indicate that they were not able to serve as a guide for migrating granule cells. The result is a profound defect of granule cell arrangement. This argument is supported by experimental studies of the role of Begmann glia in cerebellar cortex development. They indicate that developmental deficits of the Bergmann glia, alterations of their distribution and the abnormal length of their processes affect cerebellar architecture and that the severity of Bergmann glia defects correlates with misplacement of cortical cells and alterations of cortical layering (Kaartinen et al., 2001; Qu and Smith, 2005; Yue et al., 2005). In rats X-radiated neonatally (Ferguson, 1996; Li et al., 2006) and rats treated with methylazoxymethanol (Lafarga et al., 1998) or cisplatin (Pisu et al., 2005), alterations of the Bergmann radial fibers result in abnormal migration and ectopic displacement of granule cells in the molecular layer.

Lack of cerebellar cortex folding within the flocculus dysplastic area might also be a sign of a regional foliation defect. The crucial event of the initial formation of cerebellar fissures is the formation of anchoring centers at the base of developing cortical fissures (Sudarov and Joyner, 2007). In the anchoring centers, a basic role is assumed by the Bergmann glial fibers that direct migration of granule cells at the base of the fissure. PCs express and release sonic hedgehog (Shh), which regulates the number of folia via regulation of granule cell proliferation in the external granule layer (Corrales et al., 2004, 2006). Engrailed2 regulates the morphogenetic clock, which normally controls the timing of genetic and cellular events that direct formation of the anchoring centers (Sudarov and Joyner, 2007). Rat studies indicate that cerebellar cortical anomalies may also occur during postnatal life. The first signs of pathology—retraction of Bergmann radial glia, distortion of their processes and lack of endfeet—are detected in 40% of rats at 10 days of life, in the stage of active granule cell proliferation and migration (Necchi et al., 2000).

3.3. UBC abnormalities in dysplastic flocculus

The flocculus belongs to cerebellar structures, with the highest numerical density of UBCs (Diño et al., 1999). The UBC-rich region extends to the nodulus and in animals to the paraflocculus. This topography is consistent with data indicating that the flocculus extends beyond its anatomical borders to a "flocculus-like" region of the ventral paraflocculus (Tan et al., 1995). Experimental studies indicate that the flocculus and nodulus play antagonistic roles in learning and timing of eye movements (Koekkoek et al., 1997). UBCs are involved in the control of movements dependent on vestibular, proprioceptive and ocular stimuli, including vestibulooccular reflexes (VORs) controlling eye movements.

Dysplastic flocculus reveals severe deficits of calretinin-positive UBCs in autistic subjects. One may hypothesize that these developmental defects contribute to a disruption of both the structure and the function of the UBC/granule cell/PC circuits. The UBCs are excitatory interneurons located in the granule cell layer (Diño et al., 2000; Ito, 2006; Mugnaini and Floris, 1994; Nunzi et al., 2001). The majority of UBCs have a single dendritic process terminating with a compact arrangement of short dendrioles forming a brush-like structure. These cells receive glutamatergic input on the brush from an individual mossy fiber (Diño et al., 2000). Mossy fiber synapses facilitate fast and peculiarly slow currents mediated by α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid and *N*-methyl-D-aspartate (NMDA) receptors (Mugnaini and Floris, 1994; Rossi et al., 1995). The UBCs identified by calretinin immunoreactivity express the glutamate receptor subunits GluR2/3, NMDAR1 and mGluR2/3. They provide a powerful network of feed-forward excitation within the cerebellar granular layer, which may amplify vestibular signals and synchronize activity in clusters of functionally related granule cells, and patches of PCs innervated by vertical projections of granule cells (Nunzi et al., 2001). Patches of synchronously active PCs reflect the activity of underlying granule cell clusters that exert a strong excitatory drive by means of the axonal branch, which terminates on the most distal portions of the PC dendritic tree (Cohen and Yarom, 1998; Gundappa-Sulur et al., 1999; Lliňas, 1982). In dysplastic flocculus of autistic subjects, UBCs' deficits may result in the loss of synchronized excitatory drive to clusters of granule cells and disruption of granule cells' drive for patches of PCs.

3.4. Deficit of inhibitory interneurons: basket and stellate cells

PCs' function is also dependent upon their innervation by the GABAergic parvalbumin-positive basket cells and stellate cells in the molecular layer. Basket cells are located in the lower one-third of the molecular layer, and their axons contribute to the plexus surrounding PCs' soma (Sultan and Bower, 1998). Stellate cells, located in the upper two-thirds of the molecular layer, innervate PC dendrites (Palay and Chan-Palay, 1974). These two types of interneurons receive excitatory input from granule cell parallel fibers and inhibitory input from the Lugaro cells residing in the granule cell layer (Laine and Axelrad, 1998) and other basket and stellate cells (Palay and Chan-Palay, 1974). Inhibitory innervation by basket and stellate cells plays an important role in PC function; however, the reduction of PC numbers observed in the cerebellum of individuals with autism (Whitney et al., 2008)

does not affect the density of basket or stellate cells detected by immunohistochemistry for parvalbumin (Whitney et al., 2009). The deficit of basket and stellate neurons observed in the dysplastic flocculus suggests focal defects of the inhibitory system in the olivofloccular circuitry in the majority of individuals with autism.

3.5. *Abnormal serotonergic innervation of dysplastic flocculus*

Serotonergic hyper-innervation in focal cortical dysplasia indicates that developmental brain epileptogenic lesions are associated both with abnormal cytoarchitecture and altered neuronal circuitry, including focal alterations of the serotonergic fibers (Trottier et al., 1996). One may assume that focal serotonergic hyper-innervation in the dysplastic flocculus is a response to defective development of a portion of the flocculus and expresses the sprouting of serotonergic fibers to build connections with missing PCs and basket, stellate and granule cells.

The cerebellar cortex receives serotonergic innervation in the form of a plexus of fine varicose fibers that do not face any postsynaptic element. Serotonin is released through volume transmission and acts as a paracrine agent (Bishop et al., 1993; Trouillas and Fuxe, 1993). The striking increase in the number of varicose fibers in the dysplastic flocculus appears to be a sign of enhanced release of serotonin in this cerebellar structure already severely affected by developmental abnormalities. The serotonin level in the cerebellar cortex is specifically and positively correlated to the level of motor activity (Mendlin et al., 1996). Because serotonin selectively excites Lugaro cells, one may expect a modification of Golgi cell inhibitory input on large populations of granule cells, and indirectly on PCs (Dieudonne and Dumoulin, 2000). The oculomotor activity abnormalities observed in autism could be the collective product of severe changes of flocculus circuits and of exposure of this dysfunctional system to excessive amounts of serotonin.

The presence of strong serotonin transporter immunoreactivity (a) within bundles of fibers passing by the flocculus and nodulus in the cerebellar pedunculi and providing serotonergic innervation to all cerebellar subdivisions, (b) in bundles of these axons encompassing the nodulus and flocculus and (c) in bundles of these axons entering in the dysplastic flocculus suggests serotonergic hyper-innervation in the flocculo-nodular region in autism. The increased number of serotonin axons in the medial forebrain bundles, ansa lenticularis, stria terminalis, temporal superior gyrus, amygdala and globus pallidus in young autistic subjects (Azmitia et al., 2011) suggests that cerebellar regional serotonergic hyper-innervation is a component of more complex developmental alterations in autism.

3.6. *Complex nature of olivofloccular circuit abnormalities*

Flocculus dysplasia was found in eight of 12 subjects (67%) with idiopathic autism. A similar prevalence of flocculus dysplasia (6/8; 75%) was also detected in a cohort with autism caused by chromosome 15 duplication (Wegiel et al., 2012). These findings suggest that flocculus alterations are associated with autism of a different etiology.

Clinical records revealed that in the examined autistic subjects, eye contact deficits ranged across a broad spectrum. However, the strength of clinicopathological correlations is limited by partial losses of the flocculus in some cases. Moreover, postmortem examination of one cerebellar hemisphere does not preclude the presence of developmental defects in another hemisphere. In spite of these methodological deficiencies, the active avoidance of eye contact and the poor or no eye contact reported in 88% of subjects with floccular dysplasia in the examined hemisphere support the hypothesis that flocculus dysplasia in the range of 0.1–7.6% of the flocculus volume may contribute to eye contact deficits in autism.

The lack of records regarding eye contact in the control subjects with a moderate or very small dysplastic area in the flocculus precludes interpretation of the findings in this group. An intact cerebellum is a prerequisite for optimal ocular motor performance. However, cerebellum-driven motor learning promotes adaptation and compensation for lesions. Therefore, some focal cerebellar lesions do not cause eye movement alterations that strictly correspond to the defect (Kheradmand and Zee, 2011). An example of this compensatory effect is the response to flocculus-paraflocculus lesions by a significant correction of smooth pursuit deficits by PCs in the dorsal vermis and cerebellar hemispheres (Ohki et al., 2009; Xiong et al., 2010).

3.6.1. *Different role of flocculus and nodulus*

A study of 147 normal infants revealed nodular and floccular dysplasia (heterotaxia) in 14% of cases (Rorke et al., 1968); however, detection of dysplasia in a small portion of the nodulus in 75% of autistic and 80% of the control subjects in our study suggests that nodular dysplasia is a common developmental defect of the nodulus.

The role of the flocculus and nodulus is significantly different. Lesion studies reveal that the injury of the flocculus/paraflocculus impairs (a) smooth tracking of a moving target, either when the head is still (smooth pursuit) or passively moving (Belton and McCrea, 1999, 2000; Zee et al., 1981); (b) gaze-holding with the eyes drifting centripetally after eccentric eye movement, resulting in a gaze-evoked nystagmus (Kheradmand and Zee, 2011; Zee et al., 1981) and (c) downbeat nystagmus, in which eyes drift up and are brought back to the fixation target by a corrective downward saccade (Kheradmand and Zee, 2011).

The main role of the interaction of the nodulus with the ventral uvula is to contribute to the control of the rotational and translational vestibular ocular reflex (Zee et al., 2002). They act upon the “low-frequency” (sustained) component of the vestibular ocular reflex via projections to a “velocity storage” mechanism within the vestibular nuclei (Kheradmand and Zee, 2011). Lesions in the nodulus/uvula alter (a) the velocity-storage mechanisms for the horizontal vestibular ocular reflexes and spatial orientation (Cohen et al., 2002; Waespe et al., 1985) and (b) the smooth pursuit and optokinetic nystagmus. However, their exact contribution to these visual-following reflexes is unclear (Kheradmand and Zee, 2011).

3.6.2. *Role of inferior olive in olivofloccular circuit*

Olivary dysplasia in three of five subjects with autism, ectopic neurons related to the olivary complex in two subjects with autism (Bailey et al., 1998), and reduced size of the IO neurons

by 14% ($p<0.001$) (Wegiel et al., submitted for publication) suggest that IO developmental defects contribute to olivo-floccular circuit developmental defects. The flocculus is the target of the IO climbing fibers (Bastianelli, 2003; Diño et al., 1999). Experimental studies support the hypothesis that IO pathology affects PCs and that maturation of cerebellar connectivity and motor activity require a transition from multiple climbing fibers' innervation to a single climbing fiber innervation to each PC (Chen et al., 1995). Experimental lesions of IO indicate that climbing fibers control PCs' activity (Benedetti et al., 1984; McCormick et al., 1985). Climbing fibers transmit to PCs error signals that are detected by IO neurons as the difference between the intended and the executed movements (Thach et al., 1992). The cerebellar cortex is composed of distinct compartments, known as microzones (Ito, 1984; Oscarsson, 1979). PCs in each microzone receive common excitatory inputs from climbing fibers of distinct subgroup of IO neurons in the brainstem (Chen et al., 1995). However, the profound disorganization of the PC layer and the loss of PCs' basic morphological features indicate total disruption of microzones' compartmentalization within the dysplastic flocculus.

One may assume that dysplasia-related flocculus cytoarchitecture and connectivity defects are clinically expressed because they are combined with developmental defects in the IO, the second component of the olivo-cerebellar circuit, and with developmental alterations of PCs within the non-dysplastic part of the flocculus, vermis and cerebellar hemispheres. This hypothesis is supported by results of a study of the global pattern of neuronal growth defects (Wegiel et al., submitted for publication) demonstrating (a) a deficit in neuron volume in the IO by 14% in autistic subjects 4 to 8 years of age ($p<0.000$), and an acceleration of growth in subjects older than 8 years old, resulting in increase of the neuron volume to 9% above control ($p<0.000$) and (b) a 31% deficit of PCs' volume in autistic subjects 4–8 years of age, and a 17% deficit in autistic subjects older than 8 years of age. Moreover, the cerebellar dentate nucleus reveals a 25% deficit in neuron volume in autistic subjects 4–8 years of age and a 13% deficit in autistic subjects older than 8 years old.

These data suggest that in autistic subjects, the complex of (a) developmental defects of the IO, (b) profound defects of PCs; basket, stellate, unipolar and granule neurons; and

Table 3 – Tissue samples of 12 autistic and 10 control subjects, brain weight, tissue processing and embedding, cause of death.

G	Case/brain number	Sex	Age (y)	PMI (h)	Brain weight (g)	H	Fix (d)	Deh. (d)	Weight loss (%)	Emb	Cause of death
A	IBR-425-02	M	4	30	1280	R	4560	28	49	Cell	Drowning
A	AN13961	M	7	25	1610	R	330	37	55	Cell	Drowning
A	HSB-4640	M	8	14	1740	R	196	23	47	PEG	Asthma
A	AN01293	M	9	4	1690	R	1006	25	44	PEG	SUDC
A	AN11206	M	15	48	1880	R	1.733	31	–	PEG	Cardiac arrhythmia
A	AN09730	M	22	25	1375	R	1034	39	38	Cell	SUDEP
A	AN08166	M	28	43	1580	R	155	22	43	PEG	Seizure-related
A	NP06-54	M	32	–	1260	L	1139	23	42	PEG	Brain tumor
A	AN16961	M	36	24	1480	R	721	37	44	Cell	Cardiac arrest
A	AN08105	M	56	3	1570	R	692	38	52	Cell	Cardiac arrest
A	AN09714	M	60	26	1210	R	398	39	42	Cell	Pancreatic cancer
A	AN12698	M	66	13	1380	R	99	37	53	Cell	Drowning
Average				**23**	**1504**	**–**	**1005**	**32**	**46**		
SE				**4**	**60**		**352**	**2**	**2**		
C	AN2456	F	4	17	1530	R	126	41	49	Cell	Broncho-pneumonia
C	UMB-4898	M	7	12	1240	R	130	41	51	Cell	Drowning
C	UMB-1708	F	8	20	1222	R	650	36	51	Cell	Rejection of cardiac transplant
C	UMB-1670	M	13	5	1420	R	–	27	45	PEG	Hanging (asphyxia)
C	UMB-4722	M	14	20	1464	R	1067	38	44	PEG	Multiple trauma
C	UMB-1846	F	20	9	1340	R	245	37	52	Cell	Multiple trauma
C	UMB-4543	M	29	13	1514	R	89	41	49	Cell	Multiple trauma
C	UMB-1576	M	32	24	1364	R	460	37	42	Cell	Asphyxia- car accident
C	IBR-291-00	M	32	14	1401	R	50	21	–	PEG	Heart failure
C	AN11184	M	64	28	1250	R	52	37	51	Cell	Heart failure
Average				**16**	**1374**	**-**	**318**	**36**	**48**	–	–
SE				**2**	**35**		**116**	**2**	**1**		
***p*<**				**0.08**	**0.04**		**0.04**	**0.08**	**0.17**		

Group (G): Autism, A; Control, C.
PMI, postmortem interval; h, hours. H-hemisphere examined (R, right; L, left).
Fixation (Fix): duration (in days, d) of fixation in 10% buffered formalin.
Dehydration (Deh.): Duration (in days, d) of dehydration in ethyl alcohol.
Weight loss: decrease (%) of hemispheric brain sample weight during dehydration.
Embedding (Emb), Celloidin (Cell), polyethylene glycol (PEG).

Bergmann glia in dysplastic area, (c) dysplasia in the nodulus, (d) reduced size of PCs in non-dysplastic portion of the flocculus and (e) reduced size of PCs in the entire cerebellum contribute to a peculiar pattern of oculomotor system developmental alterations resulting in autistic subjects' gaze abnormalities including poor eye contact.

4. Experimental procedures

Brain hemispheric samples of 12 subjects with autism from 4 to 66 years of age, and 10 control subjects from 4 to 64 years of age were examined (Table 3). Average brain weight recorded during autopsy was 1504 g in the autistic group and 1374 in the control group ($p<0.04$). The average interval between death and tissue fixation was 23 h in the autistic group and 16 h in the control group ($p<0.08$). One brain hemisphere from each subject was fixed in 10% buffered formalin for on average 1005 days in the autistic group and 318 days in the control group ($p<0.04$). The brain hemisphere was dissected into 30-mm-thick frontal slabs and was dehydrated in a graded series of ethyl alcohol for on average 32 days in the autistic group and 36 days in the control group ($p<0.08$). Dehydration resulted in a comparable loss of weight of the hemispheric tissue sample by 46% in the autistic group and 48% in the control group ($p<0.17$). Tissue slabs were embedded in celloidin as described (Wegiel et al., 2010) or in polyethylene glycol (PEG) as described (Wegiel et al., 2012). Seven brain hemispheres in the autistic group and seven in the control group were embedded in celloidin, whereas five brain hemispheres of the autistic group and three of the control subjects were embedded in PEG. Combining the material preserved in the celloidin and PEG protocols was necessary to have a sufficient number of cerebellar tissue samples with the flocculus and nodulus region preserved for estimation of the prevalence of dysplastic changes and the link between pathology and clinical manifestations of the oculomotor system abnormalities. The cerebellum and brainstem were cut on a sagittal plane. The results of neuropathological studies of these cohorts were reported by Wegiel et al. (2010, 2012). The global characteristics of neuronal growth abnormalities in the brain of autistic and control subjects are summarized in the report by Wegiel et al. (submitted for publication).

Tissue acquisition was based on individual tissue transfer agreements with several tissue banks, including (a) the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, (b) the Harvard Brain Tissue Resource Center and (c) the Brain and Tissue Bank for Developmental Disabilities and Aging of the New York State Institute for Basic Research in Developmental Disabilities (IBR). The Institutional Review Board at IBR approved the study. Tissue samples and case clinical records were identified with a codified brain bank number.

4.1. *ADI-R and clinical characteristics*

The Autism Diagnostic Interview-Revised (ADI-R) was administered to each donor family in order to confirm the diagnosis of autism on a postmortem basis. Inclusion of a case was based on a summary of scores of four domains including qualitative abnormalities in reciprocal social interaction; qualitative abnormalities in reciprocal social interaction; restricted, repetitive and stereotyped patterns of behavior; and abnormality of development evident at or before 36 months of age. Ten subjects met the ADI-R criteria for autism (Table 4). In two other cases, inclusion was based on clinical diagnosis of autism. Four autistic subjects were diagnosed with epilepsy, and the death of one patient was seizure-related. Intellectual deficit, anxiety, aggression, self-injurious behavior and hyperactivity were reported in the majority of autistic subjects. Eye contact abnormalities (poor or absent eye contact, difficult to establish and maintain eye contact, lack of typical or reciprocal eye gaze) were reported in 9/12 cases (75%). In one case, clinical records were not available, and in the other two cases, clinical records were incomplete, and it is not known whether the subjects had eye contact deficit.

4.2. *Quantitative methods*

To detect quantitative changes, an image analyzer with a computer-controlled automatic stage installed on an Axiophot

Table 4 – Results on autism diagnostic interview – revised (ADI-R).

No.	Case/brain number	Social deficits (10)	Communication deficits		Repetitive behavior (3)
			Verbal (8)	Non-verbal (7)	
1	IBR-425-02	14	NA	10	3
2	AN13961	29	NA	14	3
3	HSB-4640	19	14	8	4
4	AN01293	26	NA	12	5
5	AN11206	29	NA	14	7
6	AN09730	28	NA	14	6
7	AN08166	22	16	NA	5
8	NP06-54	–	–	–	–
9	AN16961	23	NA	10	6
10	AN08105	19	8	13	3
11	AN09714	26	8	14	4
12	AN12698	–	–	–	–

ADI-R cut-off score shown in parentheses.

Table 5 – Antibodies used for characterization of cerebellar flocculus dysplasia.

Antibody	Target protein/cells	Host	Dilution	Pretreatment	Producer/vendor
Calbindin D-28k	Purkinje cells	Mouse monoclonal	1:1000	No	Swant; Bellinzona, Switzerland
Parvalbumin	Purkinje, basket and stellate cells	Rabbit polyclonal	1:1000	No	Swant; Bellinzona, Switzerland
Calretinin Ab-1 Clone 5A5	Unipolar brush cells	Mouse monoclonal	1:100	10 min microwaving in citric acid	Thermo Fisher Scientific; Fremont, CA
GFAP	Mature Bergmann glial cells	Goat polyclonal	1:500	No	Santa Cruz Biotechnology; Santa Cruz, CA
ST51-2 Ab45525	Serotonergic projections from raphe nuclei	Mouse monoclonal	1:300	No	Abcam Inc.; Cambridge, MA

microscope (Zeiss) and a stereology software package (MicroBrightField, Williston, VT, USA) were used.

The volume of the unaffected flocculus and the dysplastic area was determined in 12 autistic and 10 control subjects using 13 or 14 serial sections with equal distance. In both parts of the flocculus, the volume of the molecular and granule layer and white matter was estimated at 48 × magnification (objective lens 1.25 ×) using Stereo-Investigator (MicroBrightField). The border between subdivisions was verified at 184 × magnification (objective lens 5 ×). In dysplastic areas, granule layer borders were usually fuzzy as a result of loose granule cell arrangement. In affected areas, only islands of high density were delineated as a granule layer.

The volumes of the PCs' soma and nuclei were estimated in the flocculus and in the entire cerebellar cortex at 1480 × magnification on screen (objective lens 40 ×) using Nucleator (MicroBrightField). Nucleator in the entire cerebellar cortex was applied using from 4 to 6 equidistant serial sections using grid size 1800 × 1800 μm, test frame 180 × 180 × 30 μm and a mean number of counting frames of 664/case.

PCs were distinguished from other cerebellar neurons and from glial cells using histological features in CV staining including topography, spatial orientation, shape, size, prominent nucleus and nucleolus and distinct marginal and central chromatin. In the dysplastic area, misplacement, loss of spatial orientation and reduction of PCs' body size partially impeded identification, and cells with uncertain classification were omitted. Because 200-μm-thick celloidin sections are not penetrated with antibodies and sera, the PCs' morphometry was limited to CV-stained sections. To keep one standard, the dissector depth was limited to 30 μm below the top 5-μm guard zone regardless of section thickness (200 μm in the celloidin protocol, and 50 μm in the PEG protocol).

Five rays of Nucleator were used to estimate cell nucleus and cell body volume. The center of the Nucleator was located in the nucleolus. In the first approach, five intersections of Nucleator radii with the nucleus border were marked, and in the second approach five intersections with cell soma border were marked. The nucleus and soma volume were estimated from the lengths of nuclear and cellular segments of Nucleator radii. In the non-affected part of the flocculus, 397 PCs, and in dysplastic area, 130 PCs were measured on average. The mean CE was 0.004 in the non-affected area and 0.01 in the dysplastic area. The mean number of PCs examined in the entire cerebellar cortex was 253 with $CE < 0.002$.

4.3. *Immunostaining*

Several antibodies were employed to detect and characterize neuronal and glial developmental alterations in the flocculus (Table 5). Anti-calbindin mouse monoclonal antibody (mAb) D-28k was used to characterize the topography and morphology of the PC soma, dendritic tree, and axons. Rabbit polyclonal anti-parvalbumin antibody was employed to characterize the morphology of the PCs, basket cells in the internal one-third of the molecular layer, and the stellate cells in the external two-thirds of the molecular layer of the cerebellar cortex. UBCs in the granule cell layer were detected with an anti-calretinin mouse mAb Ab-1. Bergmann glia and astrocytes were immunostained with a goat anti-GFAP polyclonal antibody. Projections of the raphe nuclei serotonergic neurons were identified by using mouse mAb ST51-2 detecting serotonin transporter. Anti-mouse HRP/DAB detection kit (ab64259, Abcam Inc., Cambridge, MA) was used to enhance the immunoreactivity of the serotonin transporter-positive neuronal processes.

Serial free-floating 30-μm-thick PEG sections stored in 70% ethyl alcohol were treated in ascending concentrations of ethanol. Endogenous peroxidase was blocked with 0.5% hydrogen peroxide in absolute methanol. To block unspecific binding, sections were incubated with 10% serum in phosphate buffer solution (PBS). The primary antibodies diluted in 10% serum in PBS (as shown in Table 5) were used for sections' overnight incubation at 4 °C. The sections were washed and treated for 30 min with biotinylated species-specific secondary antibodies diluted 1:300 and then with an extravidin peroxidase (1:200) for 1 h. The reaction product was visualized with diaminobenzidine (0.5 mg/mL with 1.5% hydrogen peroxide in PBS). After immunostaining, sections were lightly counterstained with hematoxylin. Specificity was controlled on adjacent sections using the same protocol but with primary antibody.

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CHAPTER

3.9

Clinicopathological Stratification of Idiopathic Autism and Autism with 15q11.2–q13 Duplications

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GENETIC FACTORS IN AUTISM

In 1977, Folstein and Rutter (Folstein and Rutter, 1977) demonstrated a striking difference in concordance rates of autism between monozygous and dizygous twins. The studies that followed revealed close to 90% monozygous concordance rates for autism spectrum disorder (ASD) and very low concordance rates for dizygotic twins (Bailey et al., 1995), showing a significant role of genetic factors in autism etiology (Ritvo et al., 1985; Smalley et al., 1988, Steffenburg et al., 1989; Folstein and Piven, 1991; Rutter et al., 1990a, b; Lotspeich and Ciaranello, 1993). Recent studies demonstrate a contribution of both genetic and environmental factors to ASD. Liu et al. (2010) revealed 57.0% and 67.2% concordance rates for monozygotic males and females, respectively, 32.9% concordance rates for same sex dizygotic twins, and 9.7% recurrence risk for siblings, whereas Hallmayer et al. (2011) demonstrated moderate, 37% and 38% genetic heritability for autism and ASD, respectively, and 55% contribution of shared environmental factors to autism and 55% to ASD.

A genetic basis has been revealed for less than 15% of autism cases, whereas no single genetic cause explains more than 2% (Abrahams and Geschwind, 2008; Wang et al., 2009). Chromosomal abnormalities, especially large chromosomal anomalies, such as unbalanced translocations, inversions, rings, and interstitial deletions and duplications, were detected in 1.7% to 4.8% of subjects diagnosed with ASD (Lauritsen et al., 1999; Wassink et al., 2004). They are identified as duplications of 15q [dup(15)], deletions of 18q, Xp, 2q, and such sex chromosome aneuploidies as 47XYY and 45X (Gillberg, 1998; Reddy, 2005). Autism is diagnosed in 69% of subjects with maternal origin duplications 15q11.2–q13 (dup15) (Rineer et al., 1998), in 15% to 28% of individuals with fragile X syndrome (FXS) (Hagerman, 2002), and in 7% of people with Down syndrome (DS) (Kent et al., 1999).

DUPLICATIONS OF CHROMOSOME 15Q11Q13

The imprinted chromosome region 15q11q13 is known for its instability, resulting in the DNA repeats or deletions associated with several syndromes. Prader-Willi syndrome (PWS) is predominantly the result of a paternal deletion of the small nuclear ribonucleoprotein polypeptide N (SNRPN) gene in 15q11q13 (Ozcelik et al., 1992), whereas the Angelman syndrome (AS) is most often the result of maternal deletion of the ubiquitin-protein ligase E3A (UBE3A) gene (Knoll et al., 1993). Subsets of subjects with PWS or AS have been reported to exhibit autism-like behavior (Arrieta et al., 1994; Demb and Papola, 1995; Dykens and Kasari, 1997; Penner et al., 1993; Steffenburg et al., 1996; Summers et al., 1995). Chromosomal abnormalities of the proximal 15q region belong to the most common genomic aberrations detected in autistic disorder probands (Arrieta et al., 1994; Baker et al., 1994; Bundey et al., 1994; Cook EH et al., 1997; Flejter et al., 1996; Gillberg et al., 1991; Hotopf and Bolton, 1995; Kerbeshian et al., 1990; Martinsson et al., 1996; Schroer et al., 1998; Weidmer-Mikhail et al., 1998; Wolpert et al., 2000a, b). These abnormalities were found in up to 3% of subjects diagnosed with ASD. An especially strong association with autism was revealed in duplications in the range of 8 to 12 Mb derived from the maternal chromosome (Cook EH et al., 1997; Dawson et al., 2002). Interstitial triplications [int trp(15)] are relatively rare but have invariably been associated with a severe phenotype, including intellectual deficit (ID), ASD, and seizures. A few subjects have been diagnosed with duplications of paternal origin; however, they were described as clinically unaffected (Bolton et al., 2001; Cook EH et al., 1997; Mohandas et al., 1999; Schroer et al., 1998) or affected but without ASD (Mao et al., 2000; Mohandas et al., 1999). In only one subject was paternal origin dup(15) associated with ASD (Bolton et al., 2004). Because only maternally inherited aberrations of chromosome 15q11q13 have been reported to be associated with a severe clinical phenotype, one may assume that the copy number of maternal genes within this genomic region contributes to alterations of brain development and the autistic phenotype.

GENE EXPRESSION IN DUP(15)

Postmortem studies of the brain reveal that chromosome 15q11–13 duplications are associated with epigenetic alterations in gene expression that are not predicted from copy number (Hogart et al., 2008). Whole-genome expression profiling of lymphoblast cell lines derived from individuals with autism and isodicentric 15 [idic(15)] revealed 112 transcripts that are significantly dysregulated in samples from subjects with duplications. However, only four of a total of 80 genes located within the duplicated area of chromosome 15 were found to be upregulated, including an 1.5- to 2.0-fold upregulation of ubiquitin protein ligase E3A (UBE3A; 15q11-q13) and a 1.89-fold increase of HERC2. Baron et al. (2006) concluded that the majority of changes are not due to increased gene dosage in a critical chromosome 15 region, but represent potential downstream effects of this duplication, including two downregulated genes, namely, APP encoded by a gene on chromosome 21, and SUMO1 encoded by a gene on chromosome 2.

Several functional categories were identified as being associated with macromolecular catabolic processes, including the ubiquitin-dependent protein catabolism. The increase of UBE3A protein level may indicate dysregulation of ubiquitin-mediated proteasome pathway in cells with dup(15), resulting in enhanced ubiquitination of proteins for non-lysosomal degradation/disposal in response to genotoxic insult. Down regulation of SUMO1, a ubiquitin-like molecule, may indicate other forms of dysregulation of cell catabolic processes leading to decreased cell sensitivity to apoptotic stimuli and tolerance to DNA damage (Baron et al., 2006).

The γ-aminobutyric acid type$_A$ ($GABA_A$) receptor subunit genes (α5, β3, and γ3) are located in the susceptibility segment of duplicated chromosome 15 (Bass et al., 2000; Buxbaum et al., 2002; Cook et al., 1998; Menold et al., 2001). GABA is the main inhibitory neurotransmitter that binds to a complex of $GABA_A$ receptors. Polymorphisms in the $GABA_A$-β3 receptor subunit are associated with ASD (Cook et al., 1998; Martin et al., 2000). Moreover, differential methylations of the $GABA_A$ gene (Bittel et al., 2005; Gabriel et al., 1998; Hogart et al., 2007; Meguro et al., 1997) may result in epigenetic modifications and modifications of clinical phenotypes in dup(15)/autism.

CLINICAL CHARACTERISTICS

Between 1994 and 2008, approximately 160 patients diagnosed with inverted (inv) dup(15) were characterized (Battaglia, 2008; Dennis et al., 2006; Wang et al., 2004; Webb, 1994; Webb et al., 1998; Wolpert et al., 2000a, b), and had a similar male:female ratio (3:1) to that reported in idiopathic autism (Bryson, 1996) and in probands with dup (15q) (Wolpert et al., 2000a).

Clinicopathological correlations in dup(15) cohorts with considerable variations in the breakpoints, copy numbers, additional genetic and epigenetic modifications, and significant clinical differences are limited. In spite of these differences, distinctive clinical features of dup(15) syndrome, including early central hypotonia, developmental delay, intellectual disability, epilepsy, and autistic behavior, can be defined. Battaglia's summary of clinical findings (2008) suggests that:

a) in more than 75% of individuals with dup(15) hypotonia and lax ligaments, developmental delay and intellectual disability, autistic behavior, epilepsy, and minor dysmorphic features, involving mainly the face, are present;
b) from 25% to 50% of subjects are affected by brain abnormalities, growth retardation, and gastrointestinal and uninary tract defects; and
c) in less than 25% of subjects, microcephaly and congenital heart defects are observed.

Wolpert et al. (2000a) documented that multiple maternal copies of the proximal 15q region may lead to one form of autistic disorder involving genes in the 15q11q13 region. Social, communicative, and behavioral function, and many clinical features are similar among individuals with autism associated with dup(15) and those with autism arising from other causes. The authors' review shows a much greater occurrence rate of seizures in dup(15) (69%) than reported in idiopathic autism (33%) (Tuchman and Rapin, 2002; Tuchman et al., 2009), a common delay in achieving motor milestones (77%), and hypotonia (77%) in dup(15) and a much lower occurrence rate of these abnormalities in idiopathic autism. Almost all individuals with inv dup(15) have moderate to profound developmental delay and intellectual disability (Battaglia et al., 1997; Battaglia, 2008; Crolla et al., 1995; Gillberg et al., 1991; Webb, 1994; Webb et al., 1998; Robinson et al., 1993; Wolpert et al., 2000a).

Earlier studies of smaller groups of patients based on non-standardized criteria revealed autism in 33% (Leana-Cox et al., 1994) and 36% (Crolla et al., 1995) of individuals diagnosed with the syndrome. Application of standardized assessment of autistic symptoms to a cohort of 29 children and adults with dup(15) revealed that 69% of these subjects met the criteria for autism diagnosis (Rineer et al., 1998).

NEUROPATHOLOGY OF AUTISM WITH DUP(15) AND OF IDIOPATHIC AUTISM

The current knowledge of brain developmental alterations is based on the results of an examination of the brains of nine individuals diagnosed with dup(15), including seven subjects (78%) diagnosed with autism (Wegiel et al., 2012a,b). These studies demonstrate several striking differences and some similarities between subjects diagnosed with autism associated with dup(15) and idiopathic autism. These patterns indicate that the autistic phenotype might be a product of etiologically, qualitatively, and quantitatively different processes.

INCREASED PREVALENCE OF BRAIN TRANSIENT OVERGROWTH AND MACROCEPHALY IN IDIOPATHIC AUTISM AND MICROCEPHALY IN DUP(15) AUTISM

Macrocephaly, defined as head circumference greater than the 97th percentile of the normal population, has been reported in more than 20% of autistic subjects

(Bailey et al., 1995; Bolton et al., 1994; Fombonne et al., 1999; Lainhart et al., 1997). Increased brain weight was also reported in autistic subjects in postmortem studies (Bailey et al., 1993; Bauman and Kemper, 1985). A short period of increased brain size, starting before the age of 1 year (Lainhart et al., 1997; Redcay and Courchesne, 2005), results in macrocephaly in 37% of autistic children under the age of 4 years (Courchesne et al., 2001) and macrocephaly in 42% of the 19 twins diagnosed with idiopathic autism under the age of 16 years (Bailey et al., 1995). Brain overgrowth is associated with an increased number of neurons (Courchesne et al., 2011). However, approximately 15% of 2- to 16-year-old autistic children are affected by microcephaly (Fombonne et al., 1999), which is frequently associated with a more severe clinical phenotype (Guerin et al., 1996; Hof et al., 1991), including intellectual disability and other disorders (Fombonne et al., 1999).

The characteristics of individuals with dup(15) are few, but published reports show opposite proportions between macrocephalic and microcephalic subjects, unlike in idiopathic autism. In the largest examined cohort (n = 107) with dup(15), macrocephaly was detected in only 2.8%, and microcephaly in six times (16.8%) more subjects (Schroer et al., 1998). The first postmortem study of the brains of nine subjects diagnosed with dup(15), including seven subjects diagnosed with autism (78%) revealed the mean brain weight to be 303 g lower than in the idiopathic autism group ($p < 0.001$) (Wegiel et al. 2012 a, b). Age-adjusted mean brain weight for dup(15) (n = 9), idiopathic autism (n = 10) and control (n = 7) subjects was 1,171 g, 1474 g, and 1378 g, respectively. The difference between the dup(15) and control group was non-significant but suggestive ($p = 0.06$; Figure 3.9.1).

Two postmortem studies of idiopathic autism cohorts revealed epilepsy in 6 of 13 subjects (46%; Wegiel et al., 2010) and 3/10 (30%), whereas in the dup(15) group, epilepsy was diagnosed in seven of nine cases (78%) (Wegiel et al., 2012a). In the dup(15) cohort, sudden and unexplained death in patients with epilepsy (SUDEP) was diagnosed in 6 of 9 subjects (67%), and seizure-related death was determined in one case (10%), resulting in 77% of cases of sudden death (Wegiel et al., 2012a). Sudden death in the idiopathic autism cohort was reported in four of 13 subjects (31%, Wegiel et al., 2010). These data suggest that the microcephaly and very early onset of seizures are risk factors for SUDEP in the subpopulation of dup(15) subjects, and that this risk is at least twice as high in dup(15) than in idiopathic autism.

NEUROPATHOLOGICAL STRATIFICATION OF DEVELOPMENTAL ABNORMALITIES IN DUP(15) AND IDIOPATHIC AUTISM COHORTS

Applications of an extended neuropathological protocol based on examination of approximately 120 serial hemispheric sections per brain resulted in the detection of each developmental abnormality larger than 2–3 mm. Three major types of developmental change were detected, including heterotopias, dysplastic changes, and abnormal neuronal proliferation. They were found in all nine dup(15) and all 10 subjects with idiopathic autism; however, the type, topography, and number of abnormalities show significant differences between these two cohorts (Figure 3.9.2).

HETEROTOPIAS

Defects of migration resulting in heterotopias in the alveus, hilar portion of the CA3, and dentate gyrus (DG) occur very often in the dup(15) group (89%), are rare in individuals with idiopathic autism (10%, $p = 0.001$), and are not present in control subjects.

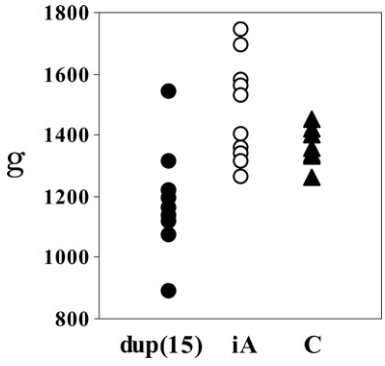


FIGURE 3.9.1 Significant reduction of mean brain weight (g) in the dup(15) autism group (1,171 g) compared to the idiopathic autism (iA) cohort (1,474 g; $p < 0.001$), and non-significant but suggestive reduction in comparison to the control (C) group (1,378 g; $p < 0.06$).

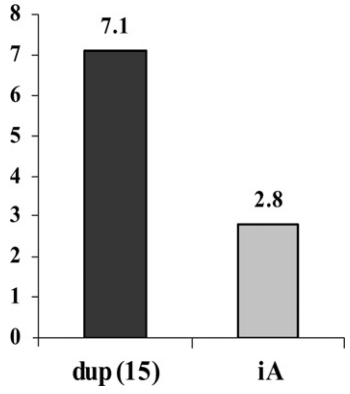


FIGURE 3.9.2 The mean number of developmental alterations detected in postmortem evaluation of serial hemispheric sections was 2.5 times higher in the dup(15) autism group (7.1/case) than in the idiopathic autism (iA) group (2.8/case).

However, the prevalence of the heterotopias in cerebellar white matter is comparable in dup(15) (56%) and idiopathic autism (60%). The heterotopias in cerebral white matter are rare in both cohorts (11% and 10%, respectively). These three patterns illustrate not only striking topographical differences in the distribution of defects of neuronal migration but also significant differences between idiopathic autism and autism associated with dup(15).

DYSPLASIA

Microdysgenesis resulting in focal developmental alterations of cytoarchitecture is also topographically selective and is detected mainly in the DG and CA fields in the hippocampal formation, and in the cerebral and cerebellar cortex. Dysplastic changes in the DG have been identified as hyperconvolution of the DG, duplication of the granular layer, massive protrusions of the granular layer into the molecular layer, focal thickening, thinning and fragmentation of the granular layer. The prevalence of dysplasia in the DG is several times higher (89%) in subjects with dup(15) than in the idiopathic autism group (10%; $p < 0.001$) (Figure 3.9.3). In the DG, usually only one type of these changes is observed in idiopathic autism, whereas from two to five types are observed in each brain in dup(15) autism. However, dysplastic changes in the CA fields are rare in both cohorts, and the percentage of affected subjects is comparable: 20% in dup(15) autism and 22% in idiopathic autism.

Another feature distinguishing these two cohorts is cerebral cortex dysplasia, which is detected in 50% of

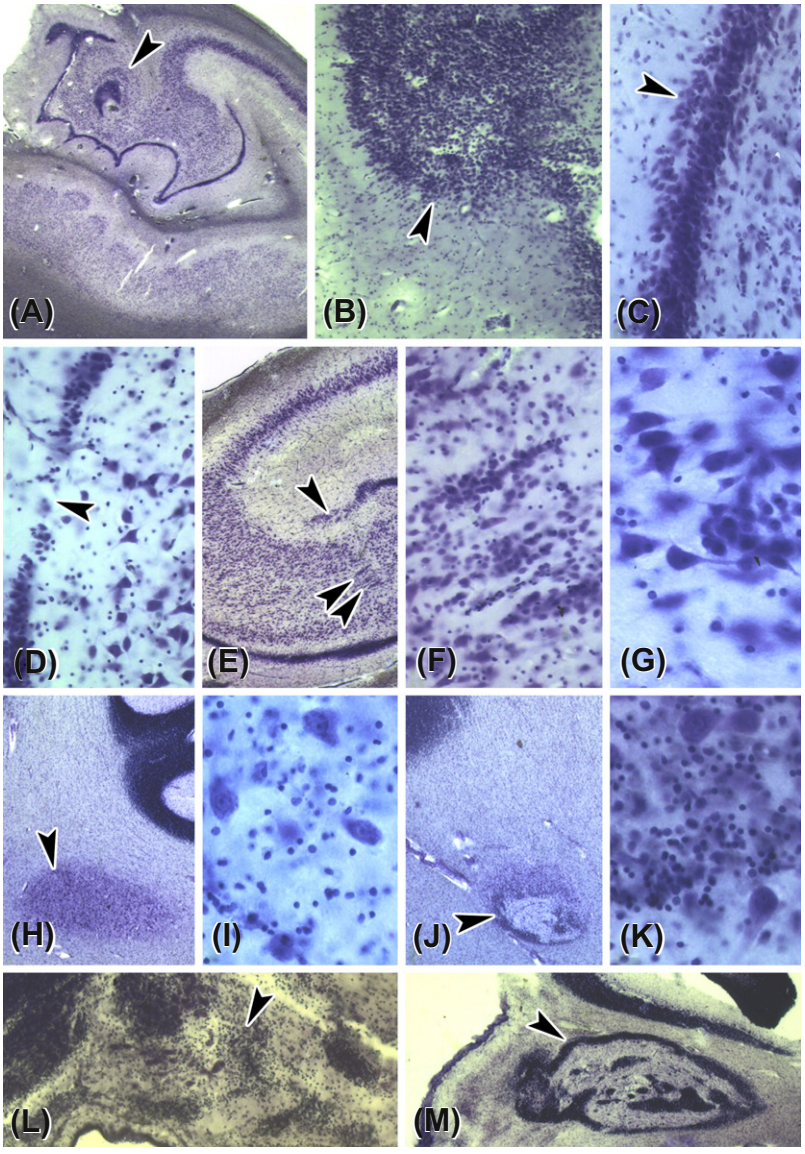


FIGURE 3.9.3 Topography and morphology of 11 types of developmental abnormalities in the brain of a 24-year-old male diagnosed with dup(15), autism, severe seizures, and SUDEP: dentate gyrus (DG) hyperconvolution and DG heterotopia in the CA4 A) DG granule cell layer protrusion (B) arrowhead, duplication (C), focal thinning and discontinuity (D), granule cell layer fragmentation (E); arrowhead; multifocal microdysgenesis within CA4 (E), two arrowheads; larger magnification of different types of microdysgenesis within CA4 (F, G); cerebellar heterotopia with morphology of cerebellar deep nuclei (H, I) and with modified morphology of cerebellar cortex (J, K); and dysplasia in the cerebellar nodulus (L) and flocculus (M).

subjects with dup(15) but is absent in idiopathic autism and in control subjects. The diversity of neocortical dysplastic changes, including multifocal cortical dysplasia with focal hypo- or acellularity, loss of vertical and horizontal organization, focal polymicrogyria and bottom-of-a-sulcus dysplasia, suggests that cortical abnormalities in dup(15) autism are the product of the disruption of several different mechanisms of cortex development.

The subependymal nodular dysplasia detected in the lateral ventricle in the occipital lobe in the brain of 15- and 39-year-old females diagnosed with dup(15) autism, and in 7- and 32-year-old males diagnosed with idiopathic autism are evidence of abnormal neuronal proliferation in some autistic subjects regardless of autism etiology and identify the predilection site for this developmental defect, which is also detectable on MRI scans (Wegiel et al., 2010, 2012a).

CAUSATIVE LINK BETWEEN DEVELOPMENTAL NEUROPATHOLOGICAL CHANGES, EPILEPSY, AND SUDDEN DEATH IN CHILDHOOD

Similar hippocampal developmental abnormalities observed in sudden unexpected and unexplained death in childhood (SUDC) cases (Kinney et al., 2007) are considered an epileptogenic focus that might be triggered by infection, fever, or head trauma and result in seizures and unwitnessed death (Blum et al., 2000; Frysinger and Harper, 1990; Yang et al., 2001). SUDC in two subjects and SUDEP in five cases with dup(15) and autism and several-fold higher prevalence of hippocampal and cortical dysplasia than in idiopathic autism appears to be the clinicopathological criterion for stratification between and within these cohorts. These developmental alterations are not pathognomonic of an 'epileptic brain' (Kinney et al., 2007), but the combination of microcephaly, the 2.5-fold higher prevalence of several types of developmental abnormalities, the 2.3-fold higher prevalence of epilepsy, and the six-times higher prevalence of epilepsy-related death in the dup(15) cohort suggests that unique genetic and molecular modifications and developmental structural defects distinguish these two cohorts of autistic subjects.

THE LINK BETWEEN DYSPLASTIC CHANGES IN THE CEREBELLAR FLOCCULUS AND ATYPICAL GAZE

Cerebellar abnormalities are among the most consistent developmental alterations detected in ASD (Bauman and Kemper, 1996; Courchesne et al., 2001; Kemper and Bauman, 1993; Ritvo et al., 1986; Whitney et al., 2008; 2009). A reduced number of Purkinje cells (PC) has been detected in 72% of the reported ASD cases (Palmen et al. review, 2004), but studies by Whitney et al. suggest that the reduced number of PC is not the effect of a developmental deficit, but is instead the result of early neuronal loss (Whitney et al., 2008; 2009). The prevalence of defective migration of cortical neurons and dentate nucleus neurons in the cerebellar white matter was almost identical in dup(15) autism and idiopathic autism (56% and 60%, respectively). Four types of cerebellar dysplasia, including nodulus, flocculus, and vermis dysplasia, and focal polymicrogyria, were found in dup(15) autism and idiopathic autism. Vermis dysplasia and focal polymicrogyria were rare in both groups.

However, a portion of the flocculus and a small portion of the nodulus, developmentally related to the flocculus ('flocculus-like' region of the ventral paraflocculus; Tan et al., 1995), were affected by dysplastic changes. Flocculus dysplasia is associated with a striking deficit of PC and unipolar brush cells, an almost complete lack of inhibitory basket and stellate cells in the molecular layer, and reduction of the number of granule cells. This abnormal flocculus cytoarchitecture appears to be an indicator of the profound disruption of the olivofloccular circuitry and severe functional alterations. The flocculus participates in the control of eye motion (Leung et al., 2000), and coordination of eye and head movements during active gaze shifts by modulating the vestibulo-ocular reflex (Belton and McCrea, 1999). Dysplastic changes are observed in the nodulus of autistic and control subjects, but the function of the affected region of the nodulus is not clear. The presence of dysplasia in the flocculus of 75% of individuals with dup(15) autism (Wegiel et al., 2012b), in 50% and 67% of idiopathic autism cases (Wegiel et al., 2012a, c), and 20% of control subjects (Wegiel et al., 2012c) and the presence of olivary dysplasia in three of five autistic subjects and ectopic neurons related to the olivary complex in two cases reported by Bailey et al. (1998) indicate that both major structural and functional components of the olivofloccular circuitry are prone to developmental defects, most likely contributing to the atypical gaze of autistic subjects. These findings also suggest that flocculus developmental defects are observed in autistic subjects regardless of etiology. Individuals diagnosed with idiopathic autism and dup(15) autism reveal altered oculomotor functions, including atypical gaze, impairments in smooth pursuit, and deficits in facial perceptions, suggesting defects in the olivofloccular neuronal circuit. These defects are reported early in the development of children with ASD (Mundy et al., 1986; Dawson et al., 1998) and contribute to deficits in using gaze to understand the intentions of other

people and their mental states (Baron-Cohen, 1995; Baron-Cohen et al., 1999; 2001; Leekam et al., 1998, 2000).

INCREASED LEVELS OF SECRETED AMYLOID PRECURSOR PROTEIN-α (SAPP-α) AND REDUCED LEVELS OF Aβ40 AND Aβ42 IN THE BLOOD PLASMA

Significantly lower concentrations of both $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$, and a reduced ratio of $A\beta_{40/42}$ detected in the blood plasma of 52 autistic children 3 to 16 years of age, compared to 39 age-matched control subjects, were attributed to the loss of Aβ equilibrium between the brain and blood (Al-Ayadhi et al., 2012). Significantly increased levels of sAPP-α in blood plasma in 60% of autistic children (Sokol et al., 2006) indicate enhanced non-amyloidogenic APP processing by α-secretase. Higher levels of sAPP-α in blood plasma were especially prominent in autistic subjects with aggressive behavior (Ray et al., 2011; Sokol et al., 2006). Enhanced non-amyloidogenic cleavage of APP with α-secretase is associated not only with ASD (Bailey et al., 2008; Ray et al., 2011; Sokol et al., 2006; 2011; Wegiel et al., 2012b), but also with FXS (Sokol et al., 2011; Westmark and Malter, 2007; Westmark et al., 2011). Due to the neurotrophic activity of sAPP-α, increased levels of this APP metabolite are considered a co-factor contributing to brain overgrowth in autism and FXS (Sokol et al., 2011). The fragile X mental retardation protein (FMRP) binds to and represses translation of APP mRNA. The absence of FMRP in people diagnosed with FXS results in upregulation of APP, $A\beta_{40}$, and $A\beta_{42}$. Similar upregulation is detected in Fmr1 knock-out ($Fmr1^{KO}$) mice (Westmark and Malter, 2007); however, genetic reduction of AβPP by removal of one App allele in $Fmr1^{KO}$ mice reverses the FXS phenotype and increases blood plasma levels of $A\beta_{1\text{-}42}$ to control levels (Westmark et al., 2011).

ENHANCED ACCUMULATION OF N-TERMINAL TRUNCATED Aβ IN NEURONAL CYTOPLASM

Neuronal proteolytic cleavage of APP by β- and γ-secretases (amyloidogenic pathway) results in release of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$, which are able to form fibrillar deposits in the extracellular space (amyloid plaques) and in the wall of brain vessels (amyloid angiopathy). $A\beta_{17\text{-}40}$ and $A\beta_{17\text{-}42}$ are products of α- and γ-secretases (p3 peptide) in the non-amyloidogenic pathway (Iversen et al. 1995; Selkoe, 2001). Aβ is generated and detected in the endoplasmic reticulum/Golgi apparatus and endosomal-lysosomal pathway (Cook D.G. et al., 1997; Glabe, 2001; Greenfield et al., 1999; Hartmann et al., 1997; Wilson et al., 1999), multivesicular bodies (Takahashi et al., 2002), and mitochondria (Bayer and Wirths, 2010; Caspersen et al., 2005). Aβ peptides differ in oligomerization and fibrillization as well as toxicity. Intraneuronal accumulation has been reported in normal human brain (Wegiel et al., 2007). Enhanced accumulation has been proposed as an early alteration in Alzheimer's disease (AD) and in transgenic mouse models of AD (Bayer and Wirths, 2010; Gouras et al., 2010; Gyure et al., 2001; Mochizuki et al., 2000; Winton et al., 2011).

Intraneuronal Aβ in human brain is mainly N-terminal truncated $A\beta_{17\text{-}40}$ and $A\beta_{17\text{-}42}$ (Wegiel et al., 2007). Cytoplasmic Aβ in neurons is a reflection of the balance between its rate of synthesis, accumulation in cytoplasmic organelles, and its degradation. The extracellular level of Aβ is a reflection of neuronal production and extracellular oligomerization, fibrilization, deposition, and disposal, including drawing of the excessive amounts through the blood-brain barrier to the blood (Weller et al., 1998). The morphology and amount of intracellular deposits of Aβ are neuron-type-specific, and show a broad spectrum of differences in developing and aging brains and in brains affected by pathology. Increased level of sAPP-α in the blood plasma of autistic subjects is linked to enhanced intraneuronal accumulation of amino-terminally truncated $A\beta_{17\text{-}40/42}$ in their neurons (Wegiel et al., 2012b).

STRATIFICATION OF Aβ ACCUMULATION IN NEURONS IN THE DUP(15) AUTISM AND IDIOPATHIC AUTISM

A postmortem study of 12 brain structures and neuronal populations (frontal, temporal, and occipital cortex; amygdala, thalamus, lateral geniculate body, DG; CA1 and CA3 fields and PC) revealed higher Aβ load in neurons in 11 subregions in dup(15) autism than in idiopathic autism ($p < 0.0001$) and in control subjects ($p < 0.0001$). In eight regions, cytoplasmic Aβ load was significantly higher in idiopathic autism than in control subjects ($p < 0.001$).

We found the excessive accumulation of Aβ in two ASD cohorts to be neuron type-specific. Classification of neuronal Aβ immunoreactivity as strong, moderate, and weak revealed two types of alterations. Type 1 is characterized by a significant increase in the percentage of neurons with strong Aβ immunoreactivity, defined as a condensed mass of indistinguishable small and large immunoreactive granules occupying a large portion of neuronal perikarya. Type 1 of Aβ accumulation is typical for the amygdala, thalamus, and lateral geniculate

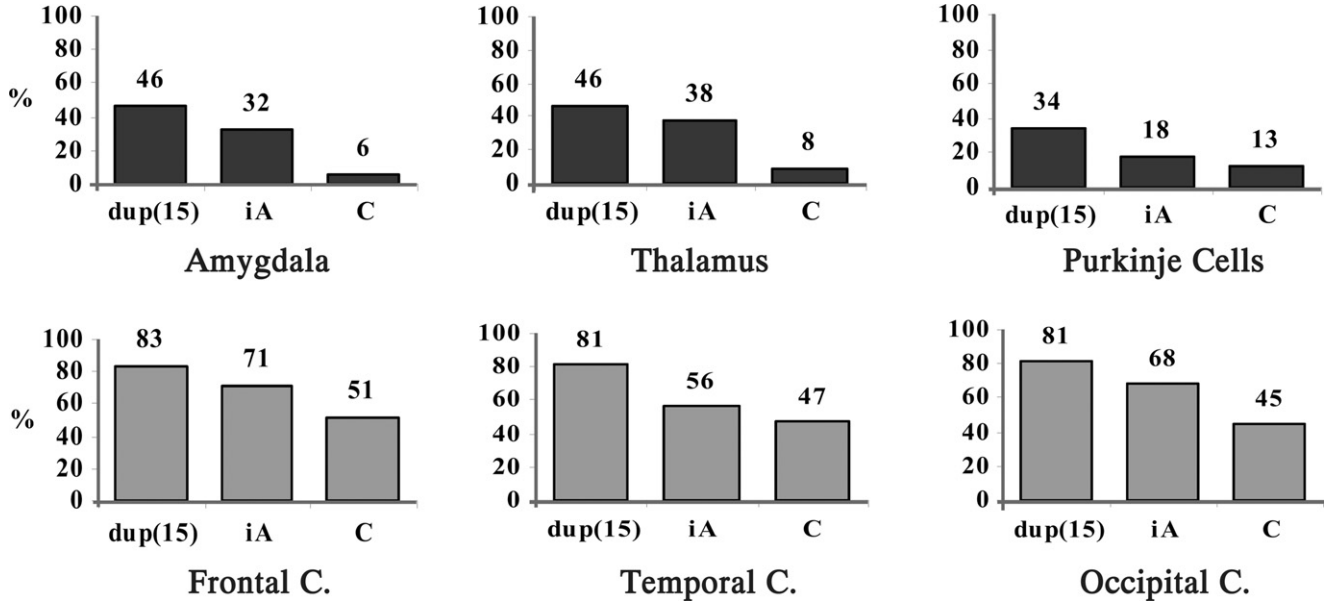


FIGURE 3.9.4 The percentage of neurons with very high intracellular Aβ load is significantly higher in the amygdala, thalamus, and Purkinje cells in dup(15) autism than idiopathic autism and control subjects ($p < 0.0001$). In the frontal, temporal, and occipital cortex, the total percentage of Aβ-immunoreactive neurons (neurons with strong, moderate, and weak Aβ immunoreactivity) is significantly higher in dup(15) autism than in idiopathic autism and control groups ($p < 0.0001$).

nucleus (Figure 3.9.4). Stratification of dup(15)autism and idiopathic autism cohorts is reflected in a 7.6-fold increase of the percentage of strongly immunoreactive neurons in the amygdala and thalamus and a 4.5-fold increase in the lateral geniculate nucleus in dup(15) autism in comparison to the control cohort. In idiopathic autism, the increase was 5.3×, 6.3×, and 3.9×, respectively, in comparison to that found in the control subjects. Type 2 of Aβ intraneuronal accumulation is distinguished by a relatively low percentage of neurons with strong Aβ immunoreactivity, but a higher total percentage of Aβ-immunoreactive neurons. This pattern was typical for pyramidal neurons in all three examined neocortical regions (frontal, temporal, and occipital cortex), and the total percentage of Aβ-immunoreactive neurons was higher in dup(15) than in the autism and control groups ($p < 0.001$).

$A\beta_{1\text{-}40}$ AND $A\beta_{1\text{-}42}$ IN DIFFUSE PLAQUES IN AUTISM

Diffuse amorphous nonfibrillar Aβ deposits are classified as preplaques (Mann et al., 1989) or pre-amyloid deposits (Tagliavini et al., 1989). However, diffuse deposits in the human cerebellar cortex and the

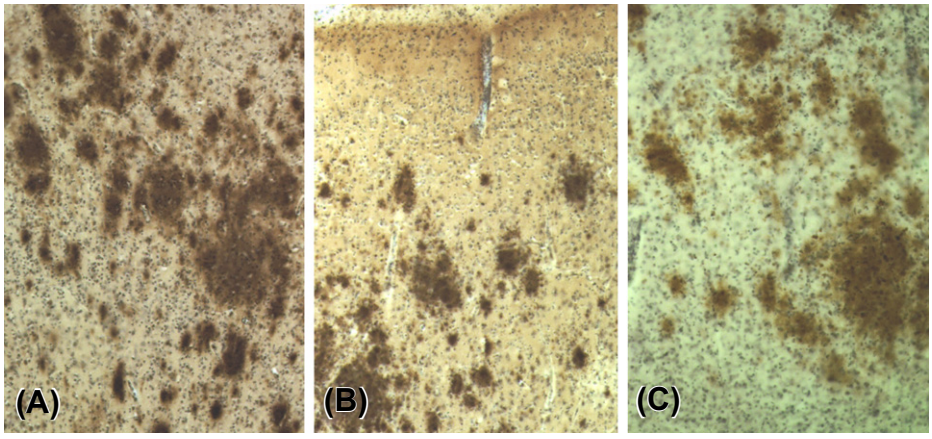


FIGURE 3.9.5 In the oldest subject examined postmortem in the dup(15) group (39-year-old autistic female), and two subjects with autistic disorder of unknown etiology (51 and 52 years old), numerous diffuse $A\beta_{1\text{-}40/42}$-immunoreactive plaques were detected in the cortical ribbon (A, B, C), respectively.

parvocellular layer of the presubiculum do not fibrilize, regardless of age or stage of AD (Wisniewski et al., 1998). Diffuse plaques were found in 2 of the 11 subjects diagnosed with ASD (51 and 52 years old) and in 1 of 9 subjects diagnosed with dup(15) and autism (Figure 3.9.5). These subjects were the oldest in both examined groups. Plaques were nonfibrillar, thioflavin S-negative, but in contrast to N-terminal truncated $A\beta_{17\text{-}40/42}$ in neurons, they contained full-length $A\beta_{1\text{-}40/42}$ (Wegiel et al., 2012b). Diffuse plaques are also observed in young people diagnosed with Down syndrome, but they are $A\beta_{17\text{-}40/42}$-immunoreactive (Gowing et al., 1994; Lalowski et al., 1996).

Both in the plaque perimeter and in plaque-free areas, numerous astrocytes and some microglial cells contain amyloid, but only N-terminally truncated $A\beta_{17\text{-}40/42}$. Focal enhanced proliferation of astrocytes, accumulation of Aβ in their cytoplasm, and accelerated death also likely results in Aβ deposition, mainly in the perivascular space.

These findings suggest that the pattern of metabolic alterations of APP processing and Aβ accumulation is comparable in the two autistic cohorts, but the severity of metabolic changes is significantly intensified in dup(15) autism in comparison to idiopathic autism. Enhanced APP processing with α- and γ-secretase increases the percentage of Aß-immunoreactive neurons and intracellular amyloid load in dup(15) autism with microcephaly. Increased levels of blood plasma neurotrophic sAPP-α detected in 60% of subjects with idiopathic autism justified the hypothesis that the increased level of the product of α-secretase may help identify a subset of children in which early brain overgrowth is sufficient for development of autism and might be a marker of the mechanism that regulates brain overgrowth (Sokol et al., 2011).

CLOSING REMARKS

Comparative postmortem studies of the brains of individuals diagnosed with dup(15) autism identify a cluster of neuropathological findings differentiating this cohort of autistic subjects with genetically identified autism etiology from a cohort of subjects with idiopathic autism. These studies support the recommendation by Happe et al. (2006) for subclassification of ASD according to different etiologies, clinical presentations, and neuropathologic findings, and most likely in terms of different preventive strategies and different treatments.

Because the complex nature of developmental abnormalities increases the risk of death in childhood and early adulthood in the dup(15) autism cohort, postmortem study appears to reflect both developmental abnormalities associated with this genetic trait, and a particular combination of factors contributing to early death. The list of factors likely related to the risk of early death and the detected pattern of neuropathological changes includes: (a) maternal origin of dup(15), (b) autism, (c) more severe clinical phenotypes with ID, early-onset and severe or intractable seizures, and increased prevalence of SUDEP, (d) high prevalence of microcephaly, (e) several-fold increase in the number of developmental abnormalities, including defects of migration and multifocal defects of cytoarchitecture, especially numerous in the hippocampal formation, and (f) several-fold increase in the percentage of neurons with increased amyloid load, reflecting enhanced APP processing in non-amyloidogenic pathway with α- and γ-secretase ($A\beta_{17\text{-}40/42}$); enhanced proliferation and activation of $A\beta_{17\text{-}40/42}$-positive astrocytes, enhanced rate of astrocytes death, and in some cases, an early onset of diffuse plaques containing $A\beta_{1\text{-}40/42}$.

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The link between intraneuronal N-truncated amyloid-β peptide and oxidatively modified lipids in idiopathic autism and dup(15q11.2-q13)/autism

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ABSTRACT

Autism is a neurodevelopmental disorder of unknown etiopathogenesis associated with structural and functional abnormalities of neurons and increased formation of reactive oxygen species. Our previous study revealed enhanced accumulation of amino-terminally truncated amyloid-β (Aβ) in brain neurons and glia in children and adults with autism. To verify the hypothesis that intraneuronal Aβ causes oxidative stress the relationships between neuronal Aβ and oxidative stress markers —4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA)—were examined in the frontal cortex from individuals aged 8–32 years with idiopathic autism or with chromosome 15q11.2-q13 duplications (dup(15)) with autism, and age-matched controls. Quantification of confocal microscopy images revealed significantly higher levels of neuronal N-truncated Aβ and HNE and MDA in idiopathic autism and dup(15)/autism than in controls. Lipid peroxidation products were detected in all mitochondria and lipofuscin deposits, in numerous autophagic vacuoles and lysosomes, and in less than 5% of synapses. Neuronal Aβ was co-localized with HNE and MDA, and increased Aβ levels correlated with higher levels of HNE and MDA. The results suggest a self-enhancing pathological process in autism that is initiated by intraneuronal deposition of N-truncated Aβ in childhood. The cascade of events includes altered APP metabolism and abnormal intracellular accumulation of N-terminally truncated Aβ which is a source of reactive oxygen species, which in turn increase the formation of lipid peroxidation products. The latter enhance Aβ deposition and sustain the cascade of changes contributing to metabolic and functional impairments of neurons in autism of an unknown etiology and caused by chromosome 15q11.2-q13 duplication.

Keywords Idiopathic autism • Chromosome-15q11.2-q13 duplication • Amyloid-β peptide • Oxidative stress • Malondialdehyde • 4-hydroxy-2-nonenal

Introduction

Autism is a complex neurodevelopmental disorder characterized by impaired reciprocal social interactions, impaired verbal and nonverbal communication, and stereotyped patterns of repetitive behavior, with clinical symptoms developing in early childhood [30]. The cause of autism is unknown, but a combination of genetic, epigenetic, and environmental factors has been proposed to play a major role in the etiology of autism. Certain genetic disorders are associated with a particularly high incidence of autism, e.g., it is as high as 69% among individuals who have a duplication of chromosome 15q11.2-q13 of maternal origin [dup(15)] [31, 48].

Altered metabolism of amyloid precursor protein (APP) in autism is indicated by higher plasma levels of secreted APP—two or more times—in children with severe autism and aggression than in children without autism, and by lower levels of amyloid-beta (Aβ) 40 than in controls [3, 45]. Hence, an increased processing of APP by alpha-secretases has been proposed to contribute to autism [37, 45]. Our recent postmortem studies demonstrated intracellular deposits of Aβ truncated on the amino-terminal side—the Aβ17-40/42—in neurons and glia in the brain cortex, cerebellum and in subcortical nuclei. The percentage of amyloid-positive neurons as well as intraneuronal Aβ load were significantly higher in individuals diagnosed with idiopathic autism and in subjects diagnosed with dup(15) and autism spectrum disorder (ASD) than in controls [50]. The pathological consequences of intracellular accumulation of N-truncated Aβ by neurons and glia are not known. However, accumulation of full-length Aβ 1-42 and Aβ 1-40 in Alzheimer's disease and Down syndrome is associated with production of reactive oxygen species and contributes to oxidative stress [5, 6, 17].

Increased formation of reactive oxygen species has been implicated in the pathophysiology of autism, and features of oxidative stress have been detected in autism, even at an early age, in peripheral tissues and in the brain. Peripheral blood in children with autism contains elevated levels of malondialdehyde (MDA), an indicator of lipid peroxidation [8], and increased levels of thiobarbituric acid–reactive substances (TBARS) in red blood cells [53]. Increased oxidative stress in children with autism was also suggested by increased nitric oxide (NO) levels in red blood cells [43] and higher urinary excretion of TBARS, lipid hydroperoxides, 4-hydroxy-2-nonenal (HNE), and

protein carbonyls along with low levels of urine antioxidants [16]. Severity of autism appears to be correlated also with urinary excretion of 8-isoprostane-F2alpha [34]. These features of oxidative stress in autism may be linked to malfunction of anti-oxidative mechanisms, as indicated by reduced serum levels of ceruloplasmin and transferrin—the major proteins with anti-oxidative properties—in the autistic group [8], as well as a significantly lower ratio of reduced/oxidized glutathione in the plasma, decreased methionine cycle turnover [23, 24], increased plasma activities of glutathione peroxidase [43], xanthine oxidase and superoxide dismutase (SOD) [53], and decreased catalase activity [53].

Oxidative stress in the brain in subjects with autism is indicated by markers of oxidative DNA damage: increased levels of 3-nitrotyrosine (3-NT) [39–41] and 8-oxo-deoxyguanosine [39]. The distribution of 3-NT indicates brain region–specific enhancement of oxidative stress, particularly in the orbitofrontal cortex, cerebellum and cortical areas involved in speech processing, sensory and motor coordination, emotional and social behaviors, and memory [41]. Other biomarkers of oxidative stress in autism—reduced glutathione (GSH) levels, and decreased ratio of GSH to oxidized glutathione in cerebellum and temporal cortex—indicate that deficits in glutathione antioxidant defense in certain regions of the brain participate in the development of oxidative stress in autism [10, 39]. The pathomechanisms causing oxidative stress in autism have not been determined; however, it can be attributed, in part, to activation of the immune system, which has been confirmed in the brain [27] and in the peripheral blood [15]. Significantly higher levels of 3-chlorotyrosine—a biomarker of a chronic inflammatory response—in cerebellum and temporal cortex may link oxidative stress in the brain in autism with a chronic inflammatory response, whereas a decreased aconitase activity in the cerebellum in autism indicates increased mitochondrial superoxide production [39].

The aim of this project was to test the hypothesis that intracellular deposits of N-truncated Aβ are not biologically neutral, but may be a source of reactive oxygen species in brain cortex neurons in subjects with idiopathic autism and in subjects with dup(15) with autism.

Materials and methods

Brain tissue

Samples of autopsy brain frontal cortex, formalin-fixed and frozen, were obtained from the Brain Bank and Tissue Bank for Developmental Disabilities and Aging, of the New York State Institute for Basic Research in Developmental Disabilities Staten Island, NY, the Harvard Brain Tissue Resource Center, Belmont, MA, and the NICHD Brain and Tissue Bank for Developmental Disorders, University of Maryland School of Medicine, Baltimore, MD (Tables 1 and 2). Idiopathic autism was confirmed by Autism Diagnostic Interview–Revised (ADI-R) score. Duplication of 15q11.2-q13 was confirmed by genotyping with 19–33 short tandem repeat polymorphisms from chromosome 15, custom and/or array comparative genomic hybridization (array CGH), Southern blot analysis of dosage with 5–12 probes, and fluorescent in situ hybridization performed using antemortem peripheral blood samples and lymphoblast cell lines [31, 48].

Immunofluorescence and confocal microscopy

Free-floating 50-μm sections of formalin-fixed and polyethylene glycol–embedded frontal cortex were used for detection of Aβ, lipid peroxidation products, and markers of cell organelles by immunofluorescence and confocal microscopy. The antibodies used are listed in Table 3. Secondary antibodies were affinity-purified donkey antisera labeled with Alexa 488 or 555 specific for the respective species (Invitrogen/Molecular Probes, Grand Island, NY, USA). Nuclei were counterstained with TO-PRO-3-iodide (TOPRO-3i) (Invitrogen/Molecular Probes). Images were collected using a Nikon C1 confocal microscope system and with EZC1 image analysis software. The images were used for further immunofluorescence quantification, basing on previous studies which have shown that measurements of immunofluorescence staining allow relative protein quantification in tissue sections when properly standardized methods are used [2]. The guidelines for proper image acquisition and controlling factors that affect the accuracy and precision of quantitative fluorescence microscopy were applied [49]. To ensure unbiased sampling of images for measurements of fluorescence intensity, sections were coded and microscopic fields in the 3rd and 5th cortical layers were randomly selected in the blue channel in

which only cell nuclei and cytoplasm were visible, and large pyramidal neurons were identified by their morphology. For measurements, confocal image layers containing cytoplasm and nucleus of large pyramidal neurons were selected. Images in three channels were collected at the amplifications at which the background staining was minimal, and the settings of channel amplification were the same for all groups tested. Specificity of immunostainings was confirmed as previously described [18–20]. The background autofluorescence for large pyramidal neurons and neuropil was measured in unstained sections and in sections stained with omission of primary antibodies. The levels of immunofluorescence intensities for Aβ, HNE, and MDA were measured using Image J software (NIH), and specific immunostainings were calculated per 1,000 pixels of cell contour or random samples of surrounding neuropil of the pixel size comparable to neurons. For each brain and each immunostaining an average of 38 cells were measured. Specific immuofluorescence was calculated after subtracting autofluorescence and nonspecific background fluorescence. Blood vessels were excluded from measurements.

Immunoblotting

Samples of frozen frontal cortex were homogenized in glass-teflon homogenizer in 10 mM TRIS buffer, pH 7.5, containing 0.15 M NaCl, 0.65% NP-40, and protease inhibitor cocktail (Roche Diagn. GmbH, Mannheim, Germany), and blood vessels and leptomeninges were removed by passing through 75μm nylon mesh. Protein contents were measured using the BCA protein assay (Pierce, Rockford, IL). Samples of brain lysates were centrifuged at 10,000 g for 10 min, and supernatants and pellets were collected. Samples containing 20 μg of protein lysate as well as supernatants and pellets obtained from 20-μg samples of full lysate were subjected to SDS-PAGE (8% tris/tricine gels). Full lysates (10 μg protein samples) were used for slot blotting. Proteins modified by MDA and HNE were detected with the antibodies listed in Table 3. The levels of actin were used as sample load controls. Densitometrical measurements of bands on membranes were performed with SigmaGel software (Jandel Sci., San Raphael, CA, USA).

Statistical analysis

For all data groups the degrees of asymmetry of the data distribution around mean values have been calculated. Because the data did not have normal distribution (measured as skewness of the distribution) natural logarithms of values were used for the Student's t-test analysis. To test specific hypotheses, pairwise comparisons were calculated using Student's t-test adjusted for the non-homogeneity of variance between two groups.

Results

Immunohistochemical reactions for Aβ

Aβ immunoreactivity was detected in neurons, glia, and in neuropil by immunofluorescence and confocal microscopy in all the control, idiopathic autism, and dup(15)/autism brain tissue samples. The intraneuronal Aβ deposits immunoreacted with two antibodies against distinct Aβ epitopes: mAb 4G8, specific for the 17-24 aminoacids of the Aβ sequence (Fig 1) and R226, specific for aa 36-42 of Aβ (not shown) but revealed almost no reaction with mAb 6E10, specific for aa 4-13 of Aβ (Fig. 1A), indicating that the deposits contained N-terminally truncated Aβ, consistent with a product of secretase-α and –γ. The N-truncated Aβ was detected in up to 60% of large pyramidal neurons in the frontal cortex in the dup(15)/autistic subjects, and in 30– 45% of neurons in idiopathic autism. Control brains contained a weaker immunoreactivity for N-truncated Aβ, which was detected in 15–35% of neurons. The intracellular Aβ-immunoreactive granules were of diameter 0.3–2.5μm, and their numbers in individual large pyramidal neurons in layers 3 and 5 varied greatly in each case in every group tested, from no reaction or a few granules, to multiple granules filling the pericaryon (Fig. 1A). The morphology of nuclei in cells without and with Aβ deposits was similar, and chromatin did not show changes typical for apoptosis. Between 10% and 50% of intraneuronal deposits of Aβ were co-localized with autofluorescent material consistent with lipofuscin granules (Fig. 1A).

To quantify the intensities of immunostaining, the specific immunofluorescence, i.e., immunofluorescence after subtraction of nonspecific background fluorescence and

autofluorescence, was measured for cells and neuropil. The average intensities of specific reactions for Aβ measured in large pyramidal neurons in the confocal image layer containing cytoplasm and nucleus were significantly higher in dup(15)/autism and in idiopathic autism than in controls ($p < 0.001$). The reactions in dup(15)/autism were significantly more intense than in idiopathic autism ($p < 0.01$) (Fig. 1B, Aβ bars). The intensities of the immunoreactions for Aβ in the neuropil did not differ between the groups studied.

Lipid peroxidation products

Immunoreactivities for HNE and for MDA were detected in all layers of the frontal cortex in all the brains examined that were diagnosed with idiopathic autism or dup(15)/autism and in controls. The immunoreactions for HNE and for MDA were detected in granules with diameter 0.25–3.5μm, located in the cytoplasm of neurons and glia, in the neuropil, and in blood vessel walls. To characterize the subcellular localization of lipid peroxidation products in large pyramidal neurons in layers 3 and 5, their presence in mitochondria, autophagic vacuoles, lysosomes, and lipofuscin was verified by confocal microscopy. Mitochondria were visualized with an antibody against cytochrome c oxidase, COX IV. The intraneuronal granules that were most intensely immunoreactive for HNE or MDA were frequently identified as COX IV–positive mitochondria in dup(15)/autism (Fig. 2) and in idiopathic autism (not shown). However, the majority of neuronal granular immunoreaction for lipid peroxidation products was not associated with mitochondria. Mitochondria in control brains contained lipid peroxidation products but the immunoreactions were much weaker than in dup(15) and autism (Fig. 2).

Autophagic vacuoles were detected with an antibody against the LC3B marker. Between 30% and 60% of LC3B-positive granules were immunoreactive for MDA (Fig. 2) and HNE (not shown) but these organelles contained no more than 5–8% of all granules immunoreactive for lipid peroxidation products in neurons.

Lysosomes, detected by the presence of Lamp-1 glycoprotein, contained granules reactive for HNE and MDA, but only about 30% of lysosomes were the site of prominent accumulation of lipid peroxidation products in autism, dup(15)/autism and controls (Fig.

2). Among the lysosomes identified by the presence of tripeptidyl peptidase I—a lysosomal peptidase with a broad substrate specificity—up to 50% contained a significant or strong reaction for lipid peroxidation products (Fig. 3, TTP).

Between 30% and 50% of large pyramidal neurons in autism and in dup(15)/autism contained intracellular granules that revealed a wide-spectrum autofluorescence detected in the red-orange, green, and deep red channels—consistent with the properties of lipofuscin. The number of autofluorescent lipofuscin granules varied from a single profile to over 20 in a single cell image. They all were immunoreactive for HNE and MDA; however, only a minority of all neuronal immunoreactivities for both lipid peroxidation products was associated with autofluorescent lipofuscin (Fig. 3).

Nuclear immunoreactions for HNE and MDA in large pyramidal neurons showed a significant variability between the cases and between individual neurons in each brain. Each brain that was tested contained large pyramidal neurons in the frontal cortex with intense as well as with scanty immunoreactions (Fig. 2–4).

Granular reactions for HNE and MDA were also located in the neuropil. Up to 15% of these HNE- and MDA-reactive profiles were co-localized with synapses, as indicated by double staining for synaptophysin (Fig. 4). The percentage of synapses that contained granules immunoreactive for MDA or HNE was 1.5–5% in dup(15)/autism and idiopathic autism and control.

Measurements of intensities of specific immunoreactivity for HNE and MDA detected in individual large pyramidal neurons in the confocal image layers containing cytoplasm and nucleus revealed significantly higher average levels in dup(15)/autism and in autism than in controls ($p<0.001$). The signals in the dup(15)/autism samples were significantly more intense than in idiopathic autism ($p<0.01$). The immunoreactions for lipid peroxidation products in the neuropil were similar in the groups studied (Fig. 1B: HNE and MDA bars).

Proteins modified with HNE and MDA revealed by immunoblotting were of the molecular sizes 50–180 kD in samples from autistic and control subjects. More than 85% of the modified proteins were soluble in low concentrations of detergent—0.65% Nonidet NP-40 (Fig. 5). None of the HNE- and MDA-modified protein bands detected in lysates,

10,000g pellets or supernatants was specific for autism. The levels of total MDA- and HNE-modified proteins detected in slot blots were similar in dup(15)/autism, idiopathic autism and in controls (Fig. 5).

The relationship between localization and levels of Aβ and lipid peroxidation products

Double immunostaining and confocal microscopy revealed that the intracellular Aβ was almost entirely co-localized with HNE and MDA in all groups, with a notable exception of the smallest granules, typically of diameter up to 0.3 μm but infrequently reaching the diameter of 1 μm, which did not reveal reactions for lipid peroxidation products (Fig. 6).

The specific immunofluorescence for Aβ and for HNE and MDA was measured in individual neurons and in neuropil. Neurons with cytoplasmic Aβ granules contained granules reactive for HNE and MDA that were more numerous and more intensely stained as compared to cells that were Aβ-negative as well as to the surrounding neuropil. This relationship was observed in all groups studied. In control brains, the granular reactions for HNE and MDA were in the majority of neurons with similar intensities and distribution as in the surrounding neuropil, and stronger immunoreactions for HNE and for MDA were detected only in neurons immunoreactive for Aβ (Fig. 7).

The neuronal reactions for HNE and MDA in idiopathic autism were stronger than in control brains (Fig. 1B, cells: HNE, MDA). The intensities of the HNE and MDA immunoreactions measured in individual cells were positively correlated with the amounts of Aβ immunoreactivity: the Pearson correlation coefficient values were $r=0.64$ ($p < 0.001$) and $r=0.74$ ($p < 0.001$), respectively (Fig. 8). In dup(15)/autism the neuronal reactions for MDA were stronger than in control brains and in idiopathic autism (Fig. 1B, cells: HNE, MDA). The intensities of the immunoreactions for MDA measured in individual cells were correlated with the amounts of Aβ immunoreactivity ($r=0.76$, $p < 0.001$) (Fig. 9). In one 11- year-old individual with dup(15)/autism, large pyramidal neurons in layers 3 and 5 with intracellular Aβ contained a strong immunoreaction for MDA close to the neuronal plasma membrane (Fig. 9, panel B). Double immunostaining for Aβ and HNE in dup(15)/autism revealed stronger neuronal HNE reactions than in

control brains and in idiopathic autism (Fig. 1B, cells: HNE). Two populations of neurons of similar numerical densities were revealed among cells with Aβ deposits characterized by distinct intracellular Aβ/HNE ratios, equal to 0.147 and 0.786 for the population with stronger and less intense immunoreactions for HNE, respectively (Fig. 9). The intensity of neuronal Aβ immunoreaction was correlated with that for HNE; the correlation coefficient in the populations with higher and lower Aβ/HNE ratio was 0.77 ($p < 0.001$) and 0.79 ($p < 0.001$), respectively.

Discussion

Oxidative stress has been detected in the brain and in peripheral organs of autistic subjects [8, 10, 39–41, 43, 53]. Our data link intraneuronal accumulation of N-terminally truncated Aβ in dup(15)/autism and in idiopathic autism to elevated intracellular levels of lipid peroxidation products, the accepted markers of oxidative stress. Analysis of the relationship between deposits of Aβ of various sizes and oxidatively modified lipids in three-color immunofluorescence revealed almost complete co-localization of intracellular Aβ with lipid peroxidation products in autism, dup(15)/autism and control brains. However, the absence of HNE and MDA in the majority of the smallest Aβ immunoreactive granules, apparently representing the earliest steps of Aβ accumulation, suggests that cellular accumulation of Aβ precedes formation of lipid peroxidation products. Hence, Aβ deposits are most likely the source of oxidative stress, rather than oxidative stress being the trigger for Aβ accumulation.

Our present and previous [50] studies revealed higher levels of accumulation of Aβ17-40/42 in neurons in individuals with idiopathic autism and with dup(15)/autism, than in controls. Detection of Aβ and its N-terminal truncation in both projects was based on immunohistochemical detection with mAbs 4G8 (17–24 aa of the Aβ sequence) but not 6E10 (4–13 aa). Even though these antibodies can recognize the epitopes in full-length APP and in APP fragments [52], in human brains fixed in formalin for at least several months, dehydrated in ethanol and embedded in polyethylene glycol the mAbs 4G8 and 6E10 do not react with APP, but detect only Aβ [18, 19, 50]. To evaluate the

amounts of Aβ and also lipid peroxidation products we applied in this report measuremets of fluorescence in digital images, instead of morphological evaluation of intensity of the immunoreaction. This new approach—quantification of relative protein amounts basing on immunofluorescence imaging—has been recently shown to be a reliable method [4]. Using known aliquots of cytochrome C embedded in gelatin these authors revealed a high accordance of the amounts of protein detected with primary and secondary antibodies and fluorescence microscopy with the actual protein levels present.

The presence of large deposits of N-truncated Aβ inside neurons suggests that the peptide is, at least in part, aggregated. N-truncation of Aβ peptides is known to enhance their aggregation and formation of the β-sheet structure [35]. The biological effects of accumulation of N-truncated Aβ are not well characterized. The peptides have neurotoxic properties—especially the Aβ 17-42 species [35, 51]—leading to apoptosis mediated mainly by the caspase-8 and caspase-3 pathways [51]. However, we did not observe apoptotic nuclei in neurons, possibly because of the young age of the subjects. Aggregated and oligomerized full-length Aβ 1-40/42 is involved in the formation of reactive oxygen species through binding transitional metals copper and iron. N-terminal truncation of Aβ lowers the ability to form reactive oxygen species, because copper is bound to His6, His13 and His14, in the N-terminal sequence of Aβ, whereas the carbonyl of alanine-2 is an oxygen ligand [17]. However, aethionine-35 can also be oxidized to form a sulfuranyl radical, which subsequently can cause lipid peroxidation [5, 6]. These data and the results presented in this study suggest that enhanced accumulation of intracellular N-truncated Aβ may result in increased production of reactive oxygen species and increased formation of lipid peroxidation products.

Our finding of higher levels of lipid peroxidation products in neurons in autism and dup(15)/autism than in controls is in agreement with the reported significantly increased levels of MDA in lysates of the cerebral cortex and cerebellum of autistic subjects [9]. The localization of lipid peroxidation products in almost all mitochondria, in some autophagic vacuoles and lysosomes, and in all lipofuscin granules most likely reflects the sites of formation of lipid peroxidation products, their intracellular trafficking, and storage of non-degradable components. Mitochondria generate the superoxide anion radical (O_2*-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO*)

in the electron transport chain reactions (review [7]). The increased levels of lipid peroxidation products in mitochondria in idiopathic autism and dup(15)/autism (Fig. 2), as well as their co-localization with N-truncated Aβ, may have very significant biological consequences for the neuron. In mitochondria, MDA has an inhibitory effect on mitochondrial complex I- and complex II-linked respiration and significantly elevates production of reactive oxygen species and protein carbonyls [28]. Thus, increased formation of HNE and MDA in neurons with N-truncated Aβ deposits may enhance the formation of reactive oxygen species in mitochondria and may be the cause of, or enhance an existing mitochondrial dysfunction in autism and dup(15)/autism. Impaired mitochondrial function has been detected in children with autism, with increased rates of hydrogen peroxide production [21], and reduced expression of numerous genes of mitochondrial complex I, III, IV, and V [1].

The presence of lipid peroxidation products in lysosomes and in all lipofuscin granules reflects the sites of processing of oxidatively modified molecules in neurons—oxidatively modified proteins and lipids are known to be partially degraded in lysosomes and deposited in lipofuscin [44, 47]. Detection of lipofuscin in brain neurons in children with autism, even younger than 10 years of age, is in agreement with the reported previously increased frequency of cells containing lipofuscin—particularly in Brodmann areas 22 and 39—in autism [29].

We have not found significantly increased levels of total HNE- and MDA-modified proteins in whole brain lysates, possibly because the Aβ-accumulating neurons constitute only a minor portion of the brain cortex, or because significant fractions of HNE and MDA detected by immunofluorescence were not protein-bound. However, lipid peroxidation products are known to modify proteins. The binding is aminoacid–specific: for HNE, it is histidine (particularly when flanked by basic amino acid residues), and less frequently Cys, and Lys. Proteins particularly susceptible to modifications by HNE include enzymes involved in glycolysis and ribosomal proteins [38]. Covalent binding of HNE to enzymes frequently causes their quick inactivation e.g., glyceraldehyde-3-phosphate dehydrogenase [20] and ion-transporting ATPases [13, 14].

Detection of HNE and MDA in the nuclei of numerous neurons points out to yet another possible biological effect of lipid peroxidation products—their influence on

transcription. MDA reacts with DNA forming guanine derivatives, which in the transcribed DNA strand of expressed genes strongly inhibit RNA polymerase II [12]. MDA can also affect transcription through modification of aldehyde dehydrogenase 2—which in the nucleus plays an important role in transcription repression through its interaction with histone deacetylases—by inhibiting its nuclear translocation and its repressive activity in general transcription [11]. The great variability of nuclear reactions for HNE and MDA (Fig. 5–7) suggests that susceptibility for this effect of lipid peroxidation products may vary among cell subpopulations.

Cellular co-localization of lipid peroxidation products and Aβ may have significant biological consequences. HNE may covalently bind Aβ at multiple locations through 1,4 conjugate addition and/or Schiff base formation, which leads to covalent cross-linking of Aβ and formation of Aβ protofibrils [42]. Furthermore, oxidative stress can even trigger deposition Aβ, as shown for cultured brain vascular smooth muscle cells [19, 20]. Accumulation of HNE may also increase neuronal levels and secretion of Aβ by up-regulating expression of BACE-1—a protease responsible for the secretase-β cleavage of APP—through activation of the c-Jun N-terminal kinases and p38 [46]. Increased Aβ levels and oxidative stress in neuronal progenitor cells may impair neurogenensis in postnatal brain. Full-length Aβ—even the less neurotoxic species 1-40—causes oxidative damage in human neuronal progenitor cells and impairs proliferation, migration, and formation of processes [32].

Analysis of the levels of Aβ and HNE in individual neurons indicates the existence of two neuronal populations in the frontal cortex with a distinct relationship between accumulation of Aβ and HNE: in one, the ratio is similar to that for Aβ and MDA; in the other, moderate contents of Aβ were accompanied by a massive accumulation of HNE. This significantly enhanced formation of HNE in a subpopulation of neurons could be related to some neuronal lineages and/or to a certain functional state of neuron. Further studies are necessary to characterize cells with these properties.

We propose that formation of deposits of N-truncated Aβ, which become a source of reactive oxygen species and lipid peroxidation products, causes neuronal dysfunction in autism. Accumulation of lipid peroxidation products causes/increases dysfunction of

mitochondria and further increases Aβ accumulation leading to a self-enhancing process. Thus, autism appears to be an Aβ-associated disorder with enhanced APP processing by secretase-α and abnormal cytoplasmic accumulation and trafficking of N-terminally truncated Aβ, which lead to enhanced oxidative stress and mitochondrial injury, contributing to abnormal neuron development and function.

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Figures

Fig.1 (**A**). Large pyramidal neurons in frontal cortex in layers 3 and 5 in a 10-year-old individual with dup(15)/autism contain granules immunoreactive with mAb 4G8, but not with 6E10, consistent with N-terminally truncated Aβ. Between 20% and 40% of intracellular granular Aβ in this brain are co-localized with autofluorescent granules of lipofuscin. *Bars* 10 μm. (**B**) Intensities of the Aβ, MDA and HNE immunoreactivities in individual neurons in the frontal cortex are significantly larger in the dup(15)/autism cases and in the idiopathic autism than in controls. The bars show average + SD. Statistical significance: * $p < 0.05$, *** $p < 0.001$.

Fig. 2 MDA immunoreactivity in large pyramidal neurons in layers 3 or 5 of frontal cortex in 10-year-old individual with dup(15)/autism [dup(15)] and in 8-year-old control subject (contr) brains. Mitochondria, visualized by immunostaining for cytochrome c oxidase COX IV, were the site of weak reactivity for MDA in control but a strong reactivity in dup(15)/autism brain. Most autophagic vacuoles (detected with antibody LC3B) and lysosomes (detected by the presence of Lamp-1 glycoprotein) did not contain a significant fraction of MDA. *Bars* 10 μm.

Fig. 3 Large pyramidal neurons in layer 5 of frontal cortex in 10-year-old individual with dup(15)/autism. Lysosomes identified by the presence of tripeptidyl peptidase (TPP), characteristic for activated cells (using mAb 8C4), were detected in cells with more intense immunoreactivity for HNE. Up to 50% of these lysosomes immunoreacted strongly for HNE. Lipofuscin, detected as autofluorescent granules in green channel, contained strong reactivities for HNE and MDA, but most lipid peroxidation products were not located in lipofuscin. *Bars* 10 μm.

Fig. 4 HNE and MDA in 23-year-old individual with idiopathic autism and in 10-year-old individual with dup(15)/autism. Between 2% and 5% of synaptic terminals (detected by the presence of synaptophysin) contained lipid peroxidation products. *Bars* 10 μm.

Fig. 5 HNE- and MDA-modified proteins detected by immunoblotting in frontal cortex lysates of autism and control brains (lanes L). More than 85% of the modified proteins were detected in supernatant (S) after centrifugation at 10,000g for 10 min, but not in the pellet (P). The levels of total MDA- and HNE-modified proteins in lysates normalized to actin levels, as detected by slot blotting, were similar in dup(15)/autism, idiopathic autism and in controls.

Fig. 6 Intracellular products of lipid peroxidation—HNE and MDA—appear as granules with diameter 0.25–3.5μm. Aβ is co-localized with intracellular HNE products with the notable exception of the smallest Aβ granules, typically with diameter less than 0.3 μm, but infrequently also larger—up to 1 μm, as shown in dup(15)/autism and control brains. *Bars* 10 μm.

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Fig. 9 Brains of individuals diagnosed with dup(15)/autism, 10 and 11 years old. Confocal microscopy revealed Aβ in more than 50% of frontal cortex neurons in the dup(15) with autism. The immunoreactivity for HNE is granular and is located in neurons, glia and neuropil. Intracellular deposits of Aβ are the sites of strong accumulation of MDA (A), but in one case of dup(15)/autism (B), large pyramidal

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Figures

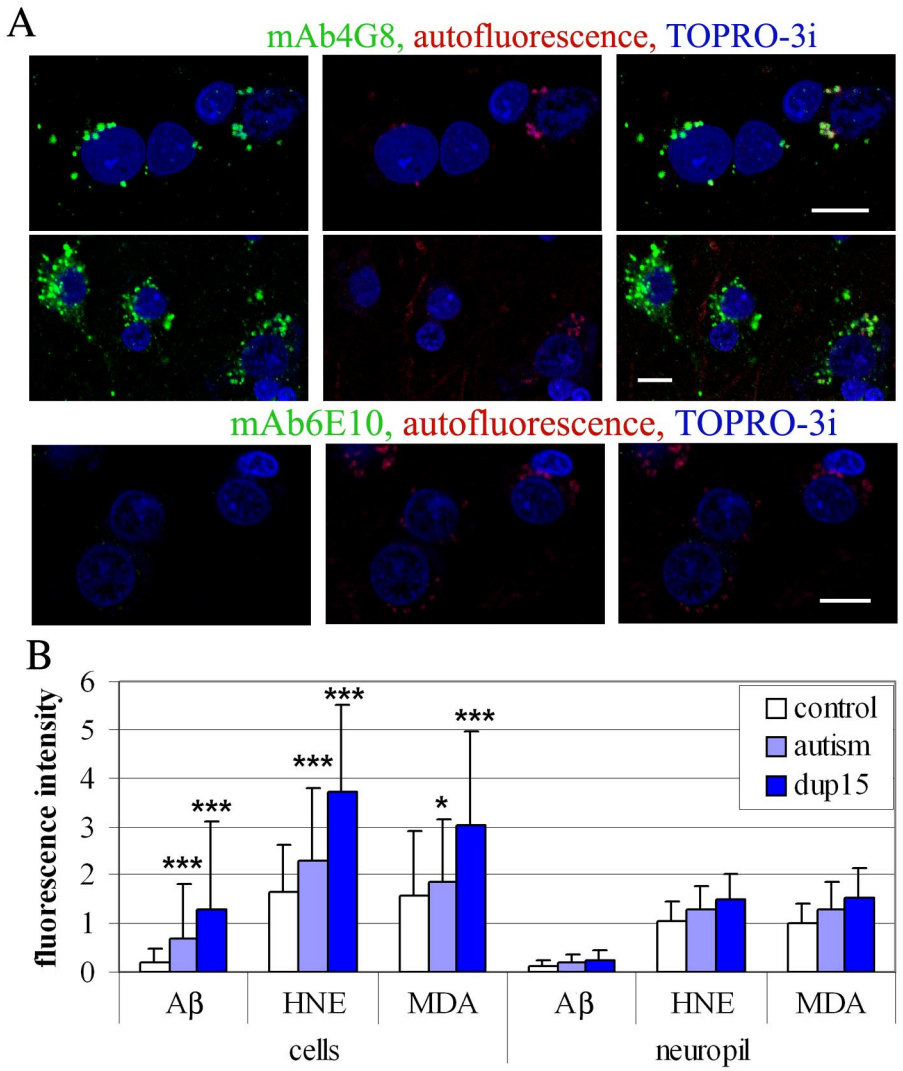


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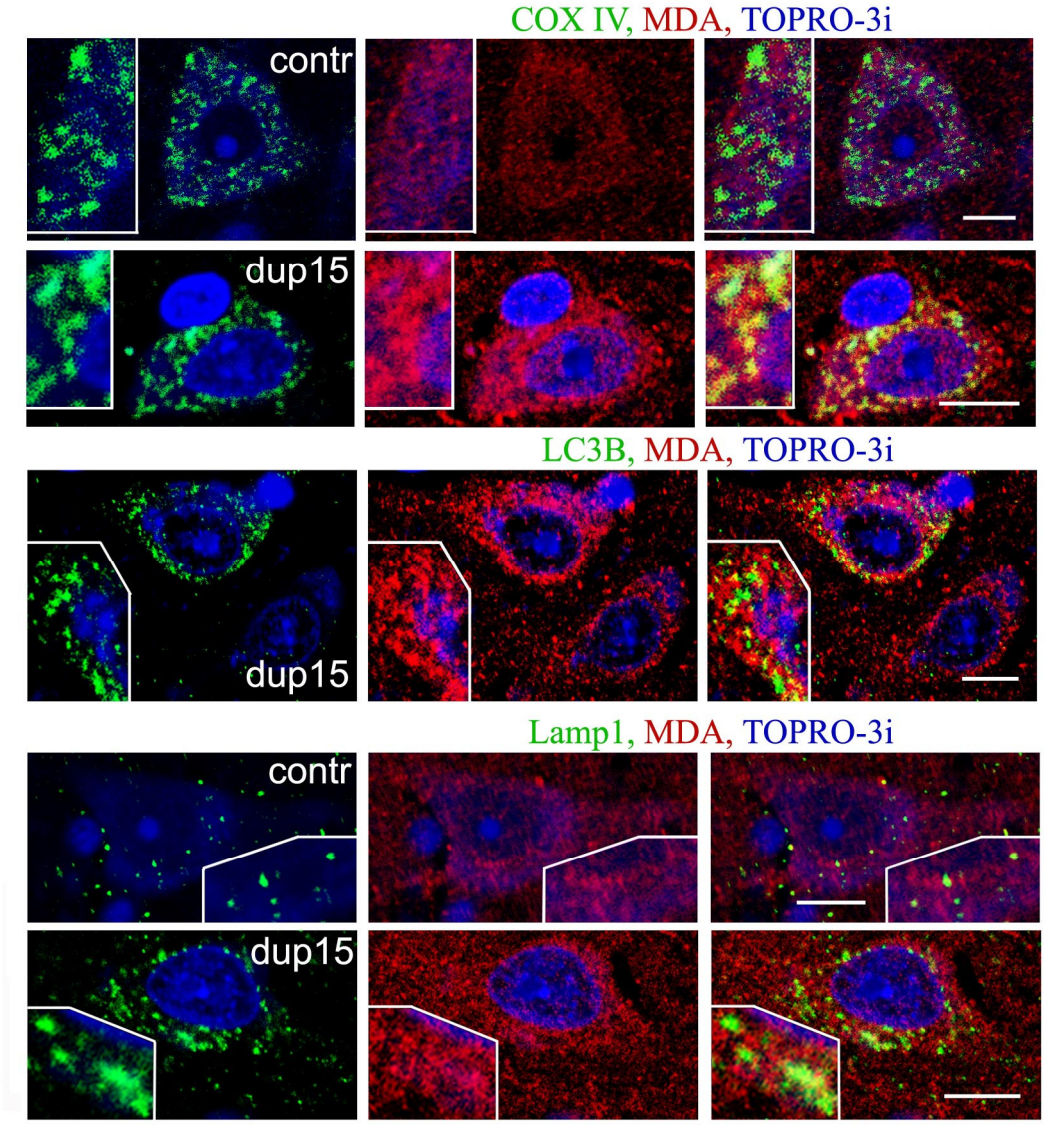


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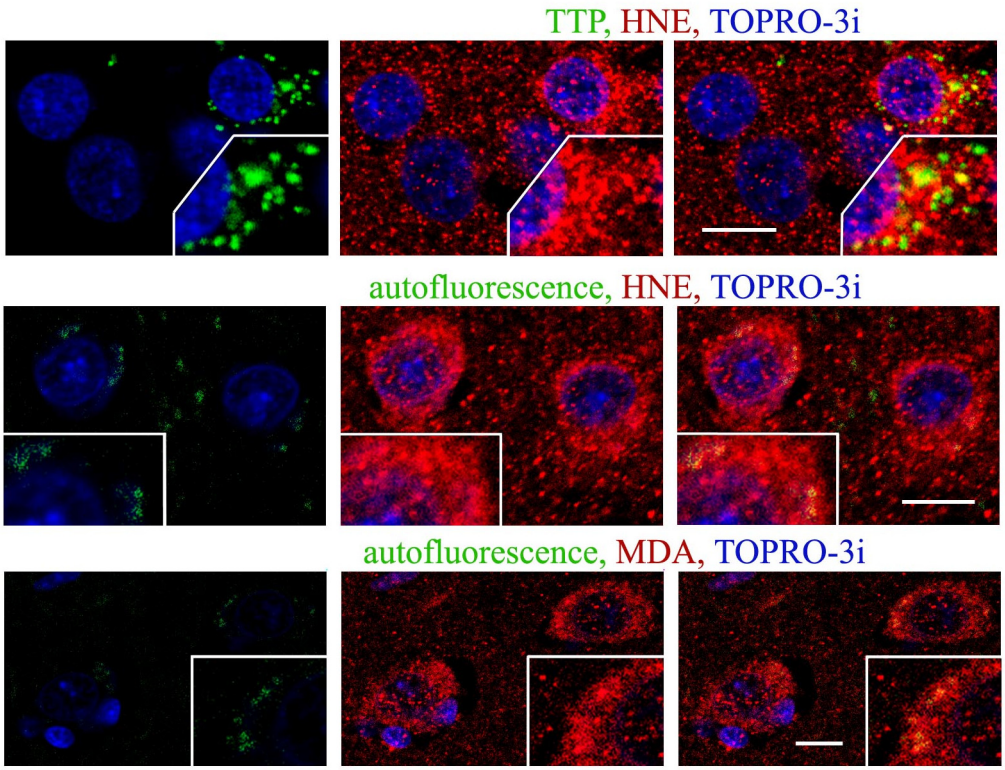


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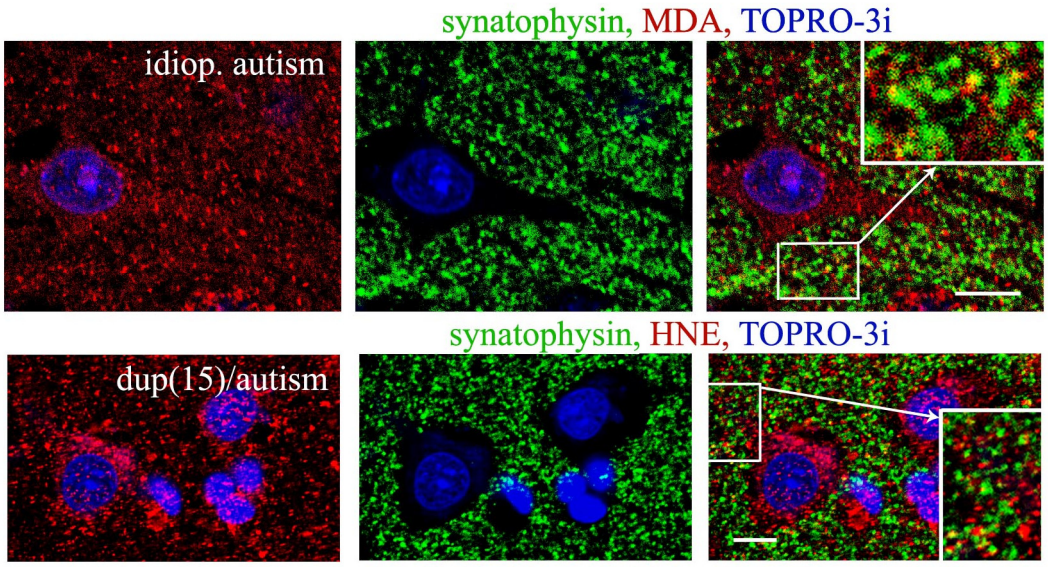


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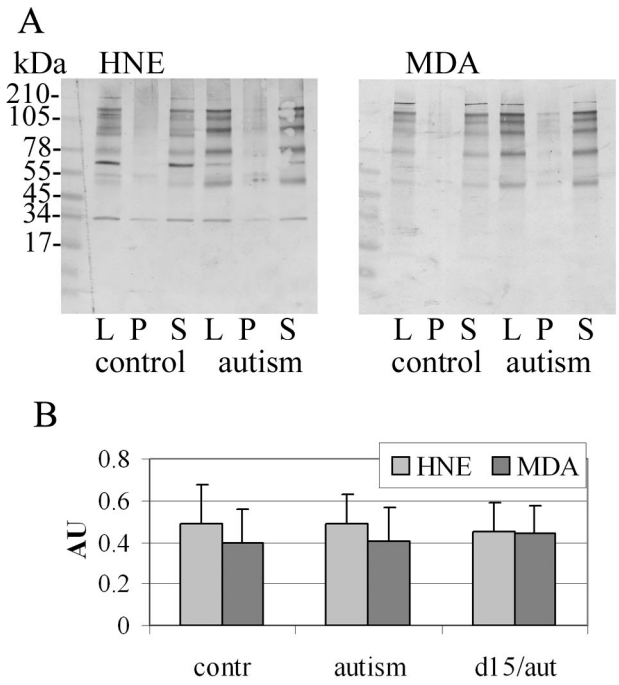


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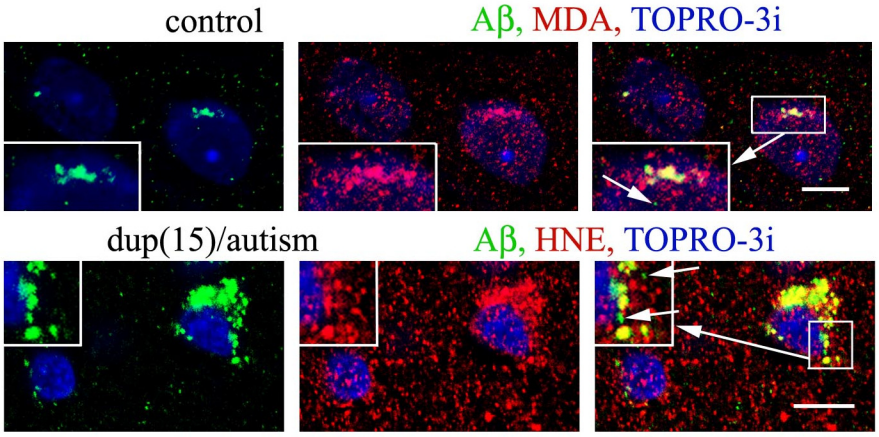


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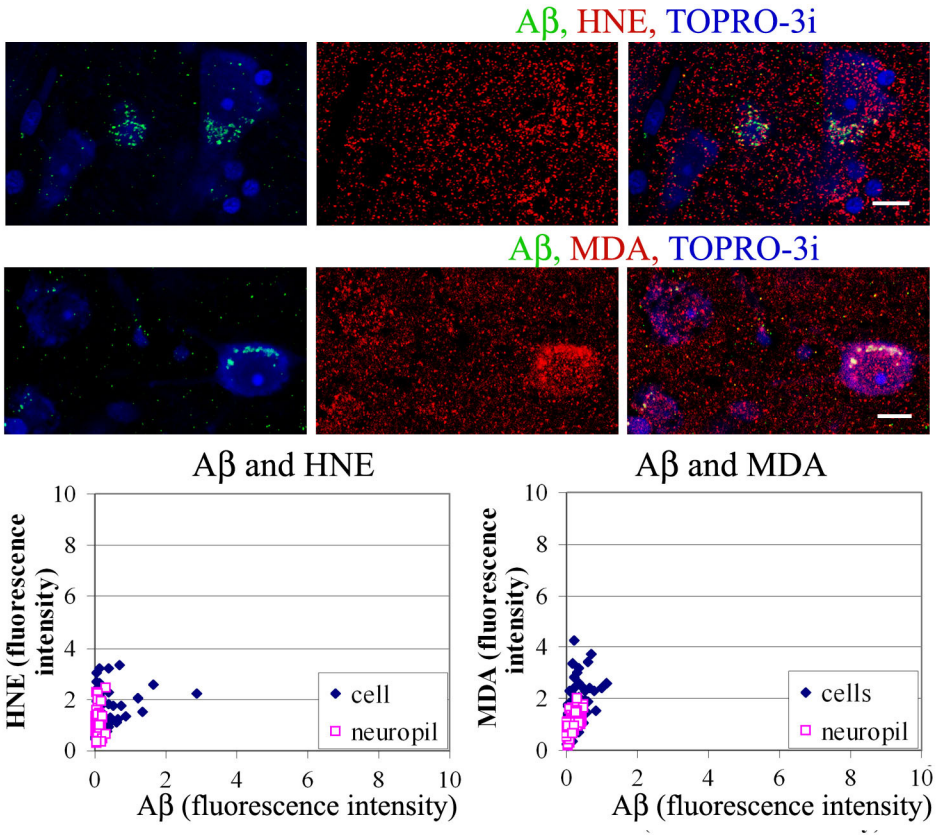


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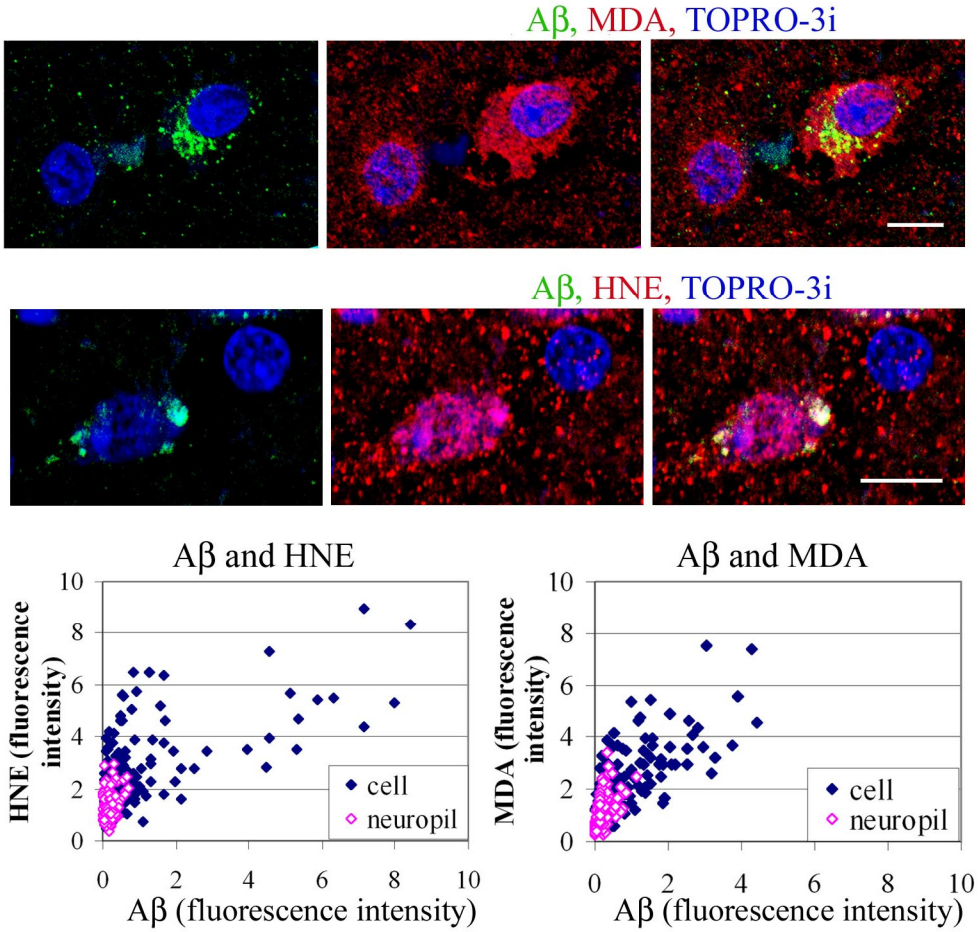


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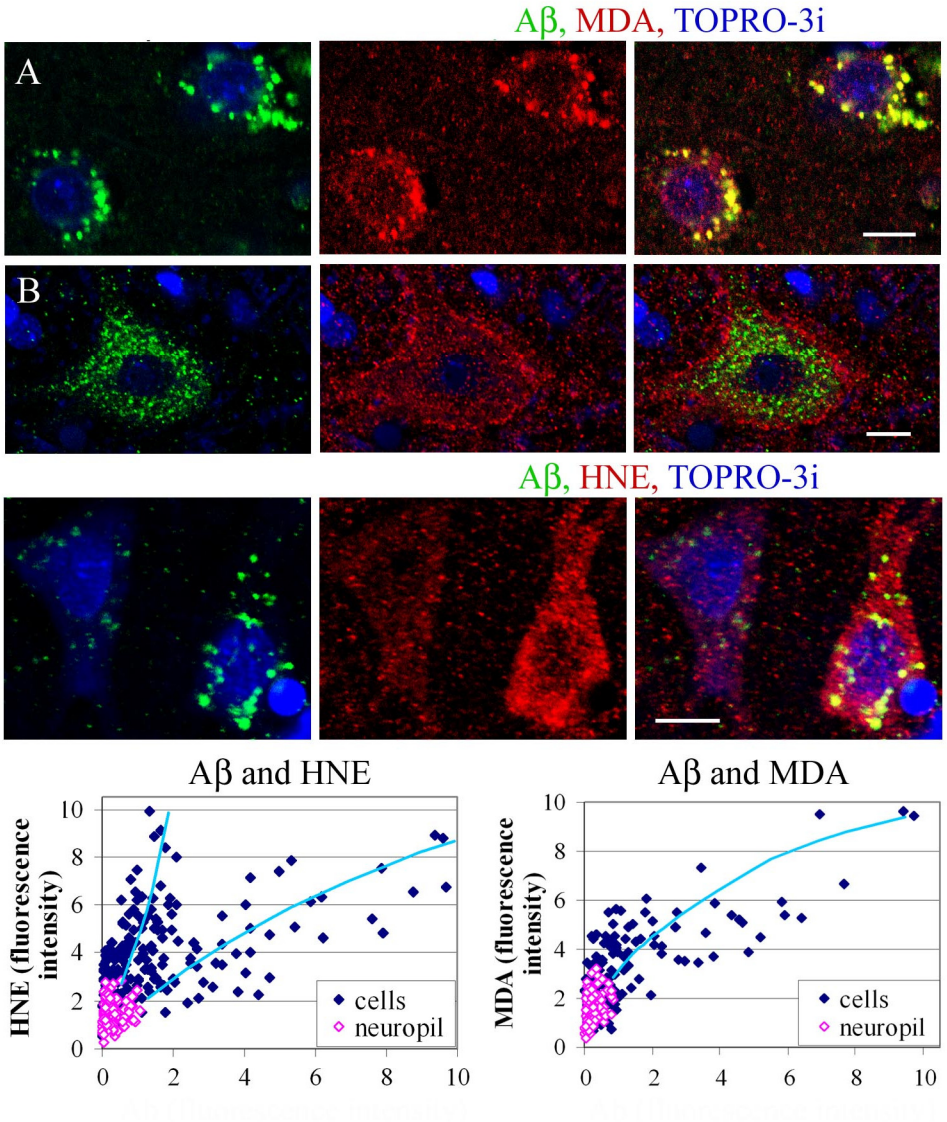


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Brain-region–specific alterations of the trajectories of neuronal volume growth throughout the lifespan in autism

Abbreviated Article Title: Trajectories of neuronal growth in autism

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Abstract

Several morphometric studies have revealed smaller than normal neurons in the neocortex of autistic subjects. To test the hypothesis that abnormal neuronal growth is a marker of an autism-associated global encephalopathy, neuronal volumes were estimated in 16 brain regions, including various subcortical structures, Ammon's horn, archicortex, cerebellum, and brainstem in 14 brains from individuals with autism 4 to 60 years of age and 14 age-matched control brains. This stereological study showed a significantly smaller volume of neuronal soma in all 16 regions in the 4- to 8-year-old autistic brains than in the controls. A very severe neuronal volume deficit seen in 12.5% of examined structures, severe in 44%, moderate in 31%, and mild in 12.5% structures, suggests desynchronization of neuronal growth in the interacting neuronal networks involved in the autistic phenotype. The comparative study of the autistic and control subject brains revealed that the number of structures with a significant volume deficit decreased from 16 in the 4- to 8-year-old autistic subjects, to 6 in the 11- to 23-year-olds-, and to 5 in the 36- to 60-year-old. Neuronal volumes in 69% of the structures examined in the older adults with autism are comparable to or significantly exceeded the size of neurons in age-matched controls. This pattern suggests defects of neuronal growth in early childhood and delayed up-regulation of neuronal growth during adolescence and adulthood reducing neuron soma volume deficit. Delayed neuron growth may contribute to age-associated clinical improvements reported in some individuals with autism. However, significant correction of neuron size but limited clinical improvements suggests that delayed correction does not restore functional deficits.

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Introduction

The clinical diagnosis of autism is currently based primarily on the observation of delayed or disrupted development of social and communication skills; of restricted, repetitive, and stereotypical patterns of behavior, interests, and activities; and having an onset prior to 3 years of age [82]. Autism does not have defining neuropathological diagnostic criteria, but smaller than normal neuron size has been reported in qualitative and quantitative studies. Bauman and Kemper demonstrated smaller neuron size and increased cell-packing density in the amygdala, medial septal nucleus, and cerebellar nuclei [12]. Casanova et al. [25, 26] reported reduced size of neurons in the frontal and temporal cortex of autistic subjects. Also, reports of a reduced perikaryon volume of pyramidal neurons in the inferior frontal cortex in Brodmann areas 44 and 45 of autistic subjects [64], and in layers V and VI in the fusiform gyrus [130] have revealed potential structural developmental abnormalities in the cortical regions involved in autism functional alterations.

All three diagnostic modalities of autism engage the majority of subcortical structures. The amygdala is involved in processing of social information and emotional interpretation, as well as fear and anxiety [6, 10, 139]. The thalamus is implicated in language functions, attention, anxiety, and obsessive thinking [95–97]. Striatal functions indicate that the caudate and putamen can be involved in repetitive motor behaviors, compulsions, and rituals [36, 107, 115]. The brainstem and cerebellar deep nuclei integrate cerebellar contributions to motor function, language, cognition, and eye motion control [79, 110]. Cholinergic neurons of the magnocellular basal complex modulate anxiety, arousal, and emotional and motor responses [68, 93]. Although several postmortem morphometric studies have documented developmental alterations in the neocortex [62, 64, 108, 119, 130], cerebellum, and brainstem [73, 74], the reported morphometric characteristics of the developmental alterations in subcortical structures are limited to the amygdala [113] and hippocampus [104]. Therefore, the first aim of this study was to test the hypothesis that developmental alterations of neuronal growth are part of a global encephalopathy of autism, including subcortical structures, hippocampus, archicortex, cerebellum, and brainstem.

Autism is viewed as a lifelong disorder whose clinical features change with development [101]. In nearly 50% of subjects later diagnosed with autism, the onset of functional alterations has been observed between 14 and 24 months, with all three diagnostic functional domains affected by age 24 months. Behavioral worsening in the second year paralleled by slowing of development [75] coincides with a rapid increase in head circumference at the age of 1 to 2 years [32, 35, 37, 38, 55]. A normal head size at birth but a larger than normal head size in 90% of 2- and 3-year-old children with autism has been reported [31]. However, a slower rate of brain growth between 2 and 4 years of age [23, 31, 55, 121] results in only a 2% brain overgrowth in adults [105] or even a smaller brain size in comparison to control

subjects [72]. The linkage between the deviation from the normal trajectory of brain growth and the severity of disease suggests a contribution of these changes to the clinical phenotype [32].

A modest improvement in the communication abilities [61, 70, 86, 94, 116], reciprocal social interactions [87, 100], and restrictive, repetitive behaviors and interests [87, 115] evident during the transition from childhood to adolescence, from adolescence to adulthood, and in adulthood helps define the behavioral trajectories of development and maturation characteristic of individuals with autism [116–118, 120]. One may assume that in autism, the age-dependent alterations of neuronal growth in the subcortical structures, the cerebellum, and the brainstem are integral components of brain pathology and reflect different clinical manifestations of autism at different stages of life. In view of this, the second aim of this study was to characterize the differences between the trajectories of neuronal growth in (a) children, teenagers/young adults, and adults diagnosed with autism, and (b) age-matched control individuals.

The brain overgrowth seen in 2- to 4-year-old children with autism and associated with overgrowth of the frontal and temporal lobes and the amygdala, but not the occipital lobe, suggests desynchronized development of the brain subdivisions. The pathological acceleration of the growth of the brain regions involved in cognitive, social and emotional functions and in language development indicates that these disproportions contribute to the functional deficits observed in autism [23, 24, 31, 55, 121]. On the other hand, neocortical studies in autism have revealed a 67% increase in the number of neurons in the prefrontal cortex [34], a 53% increase in the ratio between von Economo neurons and pyramidal neurons in the fronto-insular cortex [108], but a reduced number of neurons in the fusiform gyrus [130]. These differences suggest regional disproportions and desynchronization of brain development in autism. Therefore, the third aim was of this study was to test the hypothesis that in subcortical structures, the hippocampus, the archicortex, the cerebellum, and brainstem, the severity of neuronal growth abnormalities is region-specific.

Material and methods

Morphometric methods were applied to brains of 14 individuals with autism from 4 to 60 years of age, and 14 control subjects from 4 to 64 years of age. In the examined autistic cohort, there were 2.5 x more males than females (10 males and four females). The proportion between males and females was similar in the control group (nine males and five females).

In the first phase of this study, 39 brains were selected, including 21 from autistic subjects and 18 from controls. To reduce the risk of distorted results, strict clinical inclusion criteria and neuropathological exclusion criteria were applied. Two cases were excluded because they did not meet the Autism Diagnostic Interview-Revised (ADI-R) [82] criteria for a diagnosis of autism. In the autistic

group, one brain was excluded because of severe postmortem autolysis distorting neuronal shape and size; three brains, because of hypoxic encephalopathy; and one, because of multiple microinfarcts. In the control group, four brains were excluded because of severe autolysis. These exclusions resulted in a reduction of the autistic group by seven cases (33%) and of the control group by four cases (22%).

To confirm the diagnosis of autism, the ADI-R was administered retrospectively (Table 1). The intellectual disability of eight subjects (61%) had been evaluated with the Wechsler Intelligence Scale for Children III and the Woodcock-Johnson Tests of Achievement-Revised, and ranged from mild to severe. Seizures were reported in seven of the 14 autistic subjects (50%). In five cases, death was seizure-related (36%). Self-injurious behavior was reported in six cases (43%), aggression in four (29%), hyperactivity in three (21%), obsessive compulsive disorder in two (14%), and depression and mania in a single case for each.

Tissue preservation and morphometric methods

The postmortem interval (PMI), corresponding to the period between death and autopsy, ranged from 6 to 28 h in the control group (16.7 h on average; standard deviation [SD] 6.6 h) and from 8 to 50 h in the autistic group (21.9 h on average; SD 11.4 h). The difference in the PMI between the two groups was not significant (Table 2). Selected structures were examined in 11 right hemispheres and 3 left hemispheres in the autistic and control groups.

The average weight of the brains in the autistic cohort (1,453 g) was not significantly different from that of the control group (1,372 g). The brain hemisphere with cerebellum and brainstem was fixed with 10% buffered formalin for an average of 408 days in the control group (range 52–1819 days; SD 491 days). The average time of fixation in the autistic group was 905 days. The brains were dehydrated in a series of ascending concentrations of ethyl alcohol. The average time of dehydration was 36 and 38 days in the control group and autistic group, respectively. Dehydration was associated with not significantly different reduction of brain hemisphere weight by 47% (SD 7%) on average in the autistic group and by 45% (SD 7%) in the control group. Brain hemispheres were embedded in 8% celloidin [58]. Serial 200-µm-thick sections were stained with cresyl violet and mounted with Acrytol.

The neuropathological examination was carried out (a) to identify the type, distribution, and severity of qualitative developmental changes and (b) to eliminate brain samples affected by either pathology not related to autism, changes associated with mechanisms of death, or postmortem autolytic tissue degradation. On average, 120 cresyl violet–stained serial hemispheric sections were examined per case in blinded fashion with regard to diagnosis. The results of our study of the developmental abnormalities in these cohorts were previously reported [132]. In summary, the neuropathological evaluation revealed a broad spectrum of focal developmental alterations in the 13 examined autistic

brains, including (a) subependymal nodular dysplasia, (b) subcortical and periventricular heterotopias, and (c) dysplastic changes in the neocortex, archicortex, dentate gyrus, Ammon's horn, and cerebellum. The pathology detected in 92% of the autistic brains reflects focal modifications of neurogenesis, migration, and alterations of the cytoarchitecture. The absence of these changes in the control brains indicated that the detected alterations were autism-associated.

The morphometric measurements of the material being analyzed were performed without knowledge of the subject's age, gender, clinical diagnosis, or neuropathological status. The volume of neurons was estimated in 16 brain structures or neuronal populations, including the amygdala, entorhinal cortex, Ammon's horn, claustrum, caudate nucleus, putamen, globus pallidus, nucleus accumbens, thalamus and two parts (magnocellular and parvocellular) of the lateral geniculate body (LGB), substantia nigra, the magnocellular basal complex (MBC), including the acetylcholinergic system; Purkinje cells and dentate nucleus in the cerebellum, and the inferior olive in the brainstem.

Neuronal morphometry was performed using a workstation consisting of an Axiophot II (Carl Zeiss, Goettingen, Germany) light microscope with Plan Apo objectives 1.25x (numerical aperture, N.A., 0.15); 2.5x (N.A. 0.075), and 40x (N.A. 0.75), a specimen stage with a three-axis, computer-controlled stepping motor system (Ludl Electronics; Hawthorne, NY, USA), a CCD color video camera (CX9000 MicroBrightfield Bioscience, Inc., Williston, VT, USA), and stereology software (Stereo Investigator and Nucleator, MicroBrightfield Bioscience, Inc.). The volume of neurons was estimated with the nucleator method [50] using the MicroBrightfield software (Nucleator).

Deleted: o

Nucleator

To eliminate bias related to sectioning defects, 5-µm top and bottom guard zones were applied. To preserve the same standard of evaluation of neurons of different sizes and shapes, five rays were used in cellular measurements in all 16 regions of interest. The center of the nucleator was laid down in the nucleolus. The observer first clicked on the nucleolus and then at the five points within the focal plane where five systematic randomly rotated radii intersected with the cell border. In large neurons, radii aligned with the long axis of thick dendrites required determination of the border between cell soma and processes. For the nucleator, an abrupt reduction of the intensity of cytoplasmic staining at the base of weakly stained processes was considered to be the neuron-soma border. The soma volume was estimated from the lengths of radial cellular segments.

Neuron identification for nucleator

The neurons examined represented a broad spectrum of differences in size from the largest Purkinje cells in the cerebellum to the smallest neurons in the nucleus accumbens. In general, the neurons were distinguished from the glial cells using histological criteria provided by cresyl violet staining. The largest neurons are characterized with a low contribution of the nucleus volume to the cell body volume, whereas in smaller neurons the contribution of nucleus to cell body volume is higher. The examined neurons were larger than the glial cells, and had brain region–specific spatial orientations in examined brain structures, layers, sectors, and nuclei. Intense staining of nuclear chromatin in the small round nuclei in oligodendrocytes; small, often elongated nuclei in the microglial cells; and a round pale nucleus with a small amount of nuclear chromatin and lack of nucleolus in the astrocytes, distinguished the glial cells from neurons with a prominent nucleus and nucleolus and a distinct marginal and central chromatin.

Sampling scheme

The parameters and procedures that were applied to estimate the volume of the neuronal soma in the 16 brain structures are presented in Table 3. An optical fractionator systemic random sampling scheme was applied (Stereo Investigator, MicroBrightfield). At least four equidistant serial sections [51] were used for neuronal measures in the anatomical subdivisions of the amygdala (total 12 sections), substantia nigra (total 9 sections), Ammon's horn sectors CA1–CA4) (total 14 sections), claustrum (total 9 sections), and magnocellular basal complex (total 9 sections). In the large striatal subdivisions (caudate nucleus, putamen, and globus pallidus) with a relatively uniform cytoarchitecture, four sections were examined. Examination of the relatively large number of neurons (213/ROI/case, on average) resulted in a less than 0.01 coefficient of error (Scheaffer CE). The results of the examination of the cytoarchitectonic subdivisions of the amygdala, entorhinal cortex, Ammon's horn, and substantia nigra are presented as a mean value for the entire structures.

The grid size and the virtual counting space were designed for each brain structure individually to adjust to the size and shape of the region of interest and to reduce the variation, as reflected in the SD and the CE. The number of virtual counting spaces ranged from 34, in very small subdivisions such as the CA4 sector (CE, 0.003), to 664 for Purkinje cells, with significant differences present in the numerical density per counting space (CE, 0.002). A comparison of the results of neuronal volume estimates by different raters revealed only non-significant differences ($p < 0.87$). With the sole exception of the dentate gyrus in older subjects, controlling for seizures or SUDEP in analyses did not affect the significance of the difference in cell soma volume between autistic and control subjects.

Anatomical boundaries of examined brain structures and their cytoarchitectonic subdivisions

In this study, five components of the basal ganglia, including the telencephalic striatum (the caudate nucleus, putamen, nucleus accumbens), the diencephalic pallidum (external and internal globus pallidus), the mesencephalic substantia nigra, and the magnocellular basal complex, including the nucleus basalis of Meynert, were examined [3, 22]. They are an integral part of the cortico-subcortical circuits involved in the programming and execution of movements, and they provide the necessary timing (ramp signals) for smooth guided movements. Abnormal movements and changes in muscle tone (hyper- or hypotonia) are prominent signs of basal ganglia disorders [2, 3].

The caudate nucleus and putamen represent one cellular mass (striatum) partially separated by a thick internal capsule. The putamen extends from the external capsule to the external medullary lamina of the pallidum. In the ventral striatum, the nucleus accumbens was examined. The n. accumbens is located underneath the rostroventral aspect of the internal capsule. The border between the nucleus accumbens and dorsal striatum (caudate nucleus and putamen) was arbitrarily defined by a line perpendicular to the midline of the internal capsule at its lower end [78]. The study concentrated on the striatal small neurons, whereas a few sparse large neurons [20] were not included in this neuronal size analysis. The globus pallidus is separated from the putamen by the external medullary lamina, whereas the medial medullary lamina divides the globus pallidus into the external and internal pallidum [3]. Neurons in both parts of the pallidum were examined.

In the substantia nigra, the pars compacta was distinguished from the underlying pars reticulata by the accumulation of melanin in neuronal soma, and the nucleator examination was limited to melanin-positive neurons [21]. To identify the three major subdivisions of the pars compacta, Fearnly and Lees' [44] classification of neuronal nuclei into the dorsal and ventral tier was used. The bundle of the putaminonigral pathway was used as a marker separating the pars lateralis from the ventral tier.

In a series of CV-stained sections, four subdivisions of the magnocellular basal complex, including the most prominent nucleus basalis of Meynert [56], were identified. Four subdivisions (Ch1-4) were delineated using Vogels [131] mapping. The small Ch1 magnocellular group is located in the medial septum, whereas a substantial population of magnocellular neurons is found in the Ch2 nucleus of the vertical limb of the diagonal band of Broca. Ch2 neurons are continuous with a sparser population of large neurons that are located along the medio-ventral surface of the hemisphere in the nucleus of the horizontal limb of the diagonal band (Ch3). At the preoptic area, the magnocellular group of the basal nucleus of Meynert (Ch4 group) [89] was examined. In the amygdala, the boundaries of the lateral, basal, accessory basal and central nuclei were identified on CV-stained sections using cytoarchitectonic criteria described in details by Schumann and Amaral [113].

The mediodorsal surface of the thalamus is limited by the wall of the lateral ventricle, whereas the lateral and dorsal border of the thalamus is accentuated by the reticular thalamic nucleus that separates the thalamus from the posterior limb of the internal capsule. To obtain global characteristics of the

thalamus, which consists of more than 50 nuclei with often poorly defined borders, the entire thalamus was randomly sampled using equidistant sections.

A thin ribbon of claustrum gray matter is separated from the insula by the extreme capsule and from the putamen by the external capsule [20]. Two major anatomical subdivisions including the claustrum (compact insular claustrum and ventral claustrum), and the prepiriform claustrum were examined. Poorly defined and diffuse subregions such as preamygdalar claustrum [85], were excluded from this study.

Purkinje cells were identified on the basis of their distribution along the border between the molecular and granular layer, their large soma size, and their apical dendrite spatial orientation. The study of deep cerebellar nuclei was limited to neurons within the dentate nucleus to eliminate random contributions of neurons of the small and medially located fastigial, globose, and emboliform nuclei.

The study of the inferior olive, divided into a principal nucleus, dorsal and medial accessory olives, dorsal cap of Kooy, ventrolateral outgrowth, and nucleus β [77], was limited to the largest principal nucleus.

The entorhinal cortex (EC, Brodmann area 28) spreads over the gyrus ambiens and the anterior portion of the hippocampal gyrus. The anterior limit of the EC starts about 5 mm rostrally to the amygdaloid complex. The EC ends anterior to the rostral pole of the lateral geniculate body. Rostrally, the medial portion of the EC borders with the preamygdaloid cortex in the area marked by the sulcus semiannularis. Caudally, the median portion of the EC borders with the subicular complex. The lateral border is distinguished as the border with Brodmann area 35 that lacks a distinct layer IV [5]. The transentorhinal zone [19] was not included in this study.

Neurons were measured within four layers of the EC. Layer II consists of islands of relatively large modified pyramidal and stellate neurons separated by an acellular gap from layer III [19]. A broad layer III is built up of medium-sized pyramidal neurons separated from layer V by an acellular lamina dissecans labeled as layer IV. Layer V consists of the sublayer Va with large pyramidal neurons, Vb with smaller and more loosely arranged neurons, and sublayer Vc with relatively few dispersed neurons. Layer VI is built up of the smallest neurons, and the border of this layer in the rostral portion is characterized by a decreasing gradient of neuronal density, whereas in the caudal portion, the border is sharp [5].

Ammon's horn neurons, in the pyramidal layer sectors CA1–4, were examined. The CA1 sector pyramidal neuronal layer extends from the subiculum to the CA2 sector as a thick band of relatively small pyramidal neurons. The border between the CA1/CA2 sector and CA2/CA3 sector is accentuated by a compact arrangement of large pyramidal neurons in CA2. Moreover, the pyramidal layer is narrowest in CA2. The size of neurons in the CA3 and 4 is similar, but parallel arrangement of neurons in the CA3 sector distinguishes CA3 from the hilus neurons (identified also as CA4 sector) [40, 84]. The mapping of

sectors on serial sections representing the entire rostro-caudal extension was based on the Amaral and Insausti [5] classification, but with the CA4 identification as a separate subdivision.

A six-layered lateral geniculate nucleus (LGN) borders at the base (hilus) [47] with the transverse fissure, whereas the posterior limb of the internal capsule separates the LGN from the lateral wall of the thalamus. The major portion of the LGN is present on the level of the border between the head and body of the hippocampus. Neurons were measured separately in two major ventral laminae (1 and 2; magnocellular LGB) and in the parvocellular laminae (3 to 6; parvocellular LGB), with neurons approximately half the size of neurons in the magnocellular laminae.

Tissue and medical records were handled in accordance with the NIH Guide for the use of human tissue. The research project and protocols were approved by the Institutional Review Board of the New York State Institute for Basic Research in Developmental Disabilities (IBR). The tissue was obtained from the Harvard Brain Tissue Resource Center, the Brain and Tissue Bank for Developmental Disorders of the National Institute of Child Health and Human Development at the University of Maryland, the Brain Bank at IBR, Staten Island, NY, and the Mount Sinai Medical School, Bronx, NY. Tissue samples were coded with the number assigned by the brain banks, and this ID was the only identifier of the tissue, MRI scans, and clinical records. The Autism Tissue Program (Autism Speaks, Princeton, NJ) provided access to brain tissue samples and to the database with coded and anonymous characteristics of the autistic subjects, including the subject's age at time of death, clinical diagnosis, results of application of the ADI-R, cause of death, PMI, and fixation time.

Statistical analysis

This study analyzed the effect that autism has on neuronal soma and nuclear volume, with age-matched controls as a comparison group. Data have been post-stratified to adjust for the differing numbers of neurons sampled per individual so as to weight each individual equally. Any data points lying more than 1.5 times the interquartile range below the 25^{th} percentile or an equal amount above the 75^{th} percentile for each structure or subdivision were considered outliers and omitted from analyses. Removed defective records accounted for 0.3% of all records.

Analyses were carried out separately for three groups of cases: 4 to 8 years, 11 to 23 years, and 36 to 60 years of age. Three groups of controls were selected to provide age-matching at the group level. The age ranges for control groups were 4 to 8 years, 14 to 23 years, and 29 to 64 years of age. Comparisons were carried out across 16 regions. Adjustment for multiple comparisons was made using the Benjamini-Hochberg method [16] to maintain a False Discovery Rate (FDR) of 0.05. Accordingly, p values of 0.027 or less were considered statistically significant. Analyses were conducted using versions 11.1 and 12.1 of the Stata statistical package [123, 124].

Adjusted mean volumes and standard errors were computed using the multilevel sampling survey data procedures of the Stata package. Preliminary analyses regressed neuronal size, normalized by expressing each neuron's size as a proportion of the size of the mean control neuronal size for that structure, on PMI (in hours), fixation time (in days), brain weight (in grams), brain weight loss during processing and dehydration (as a percentage), duration of dehydration (in days), autism status and log age (in years). This analysis resulted in the detection of significant effects for each of these factors and potential confounders of autism's effects on each of these factors. Age and potential confounders of autism's effect on neuronal size accounted collectively for 1.09% of the variance in size. Autism status, when entered into the model, raised the explained variance to 1.91%. As autism status univariately explained 1.45% of variance, all potential confounders considered together diminished the variance explained by the autistic status by approximately 43%, while autism remained by a large margin the strongest predictor in the model.

Further analyses of the potential effects of confounders, including analyses of the effect of a history of seizures and of sudden and unexplained death in patients with known epilepsy were performed using the Stata svy:regress command, with potential confounders and autistic status (or age group) entered as predictors of neuronal or soma volume. Principal analyses were conducted using mixed models in which neurons were the primary unit of analysis and autistic status was entered as a second level fixed factor. This method takes into account the variance between neurons while assessing the differences between brains of autistic and control individuals. PMI, fixation time, brain weight, brain weight loss during dehydration, and duration of dehydration were entered as covariates in all analyses.

Results

Region-specific neuronal volume deficit in autistic 4- to 8- year-old subjects compared to age-matched control subjects

The 16 brain regions examined represent a broad spectrum of brain structure– and neuron type–specific differences in neuronal soma size (Fig. 1). In the 4- to 8-year-old control subjects, the range of volumes of the examined neurons extended from very large neurons such as Purkinje cells (11,635 μm^3), dopaminergic neurons in the substantia nigra (9,008 μm^3), and neurons in the magnocellular basal complex (8,385 μm^3) to very small neurons in the striatum, including the putamen (1,316 μm^3), caudate nucleus (1,199 μm^3), and nucleus accumbens (1,181 μm^3) (Fig. 2, Table 4).

Neuropathological evaluations of the CV-stained sections revealed that the neurons in the majority of the examined regions in 4- to 8-year-old autistic subjects were smaller compared to those in

age-matched controls (Fig. 1). However, in older subjects, the difference was less marked or was undetectable in the majority of examined regions.

The application of the nucleator to estimate the difference between neuronal soma volumes in four autistic and four control subjects 4 to 8 year of age revealed a significantly smaller volume of neurons in all 16 brain regions (FDR<0.05) in the autistic subjects (Table 4). However, the reduced volumes of the neuronal soma varied within the examined regions over a very broad range from 5% to 34%. An arbitrary categorization of the severity of the developmental deficits identified two brain regions (12.5%) with a very severe (> 30%) neuronal volume deficit, seven regions (44%) with a severe (22%–29%) deficit, five regions (31%) with a moderate (12%–20%) deficit, and two regions with a mild (5%) volume deficit (Fig. 2). A very severe volume deficit was found for the nucleus accumbens (-34%) and for Purkinje cells (-31%). A severe volume deficit was detected for the claustrum (-29%), thalamus (-27%), dentate nucleus (-25%), amygdala and entorhinal cortex (-24%), magnocellular basal complex and caudate nucleus (-22%). A moderate volume deficit was detected in the globus pallidus (-20%), putamen (-17%), inferior olive (-14%), Ammon's horn and magnocellular lateral geniculate body (LGB) (-12%). A mild, but still statistically significant, deficit was found for the parvocellular LGB (-5%) and the substantia nigra (-5%). The average neuronal soma volume deficit for 16 brain structures in the 4- to 8-year-old children with autism was 20%.

The pattern of significance of the neuronal size differences between autistic and control subjects was not modified when regression analyses were controlled for potential confounders, including PMI, fixation time, time of dehydration, and brain hemisphere loss of weight due to dehydration in the 4- to 8-year age group. Small modifications of significance were observed in a few brain regions in the older cohort. Corrected significances are shown in Table 4.

Fig. 3 illustrates the large contribution of smaller neurons in the autistic 4- to 8-year-old subjects in comparison to the controls and the partial or almost complete overlap of the distribution curves in the 11- to 64-year-old subjects. In general, this pattern was similar for large neurons (Purkinje cells and neurons in the magnocellular basal complex), medium-size neurons (thalamus, and amygdala); and small neurons (caudate nucleus and nucleus accumbens).

Region-specific trajectories of age-associated changes of neuronal volume in the autistic cohort in comparison to controls

To detect the age at the major increase in the volume of neurons in the autistic subjects, the older than–8 years cohort was divided into two age groups, including six autistic subjects from 11 to 23 years of age and four from 36 to 60 years of age. Age-matched control groups consisted of four subjects from 14 to 23 years of age and six subjects from 29 to 64 years of age. The study revealed three region-specific trends: (a) persistent neuronal

volume deficits in all three age groups, (b) increase of neuronal volume to control levels, or (c) increase of neuronal volume to above control levels.

While in the 4- to 8-year-old autistic subjects significant volume deficits were present in all 16 subregions, the comparison of neuronal volumes in the autistic and control subjects from 11 to 23 years revealed significant volume deficits (mean of 15%) in only 6 of 16 examined regions (Table 4, Fig. 4). In the autistic subjects older than 36 years of age, significant neuronal volume deficits (mean of 9%) were present in only five regions. This analysis identified the Purkinje cells and neurons in the claustrum, amygdala, and the Ammon's horn as one fourth of the examined subregions with persistently lower neuronal soma volumes in the autistic subjects compared to controls during the entire lifespan. In four structures of the striatum (caudate nucleus, putamen, globus pallidus and nucleus accumbens) and in the dentate nucleus and parvocellular LGB, the difference between neuron volume in autistic and control adults became insignificant, whereas the volume of neurons in the thalamus, entorhinal cortex, inferior olive, magnocellular LGB and in the substantia nigra exceeded significantly (by 8% on average) the neuron size in control adults. These data indicate that neuronal volumes in 69% of the structures in from 29 to 64-year-old autistic individuals were comparable to or significantly exceeded the size of neurons in age-matched controls.

Differences between the trajectory of neuronal volume changes within the autistic and control cohorts during the lifespan

The previous analysis characterized the relative age-associated changes of neuron soma volume in autistic subjects in comparison to controls. The aim of the second analysis was to characterize independently the trajectory of neuronal volume changes within the autistic cohort and within the control cohort. This analysis revealed opposite trends, with an increase in neuronal volumes in both the second and third age groups of autistic subjects, and a decrease of neuronal sizes in the majority of regions examined in older control groups (Table 5, Fig. 5).

In autistic teenagers/young adults (11–23 years of age), a significant increase ($p < 0.02$) in neuronal volumes (in comparison to the 4- to 8-year-old children with autism) was detected in nine regions, including the nucleus accumbens, 31%; claustrum 15%; thalamus, 14%; amygdala, 16%; entorhinal cortex, 11%; magnocellular basal complex, 23%; caudate nucleus, 27%; inferior olive, 9%; and magnocellular LGB, 17%; with a mean neuron volume increase by 18%. Further significant increase of neuron soma volume was observed in autistic subjects 36-60 years of age in six brain structures, including: thalamus, 16%; entorhinal cortex, 21%; caudate nucleus, 2%; Ammons horn, 10%, and in the substantia nigra, 22%, with a mean neuron volume increase of 14%. In autistic adults reduced neuron soma volume ($p < 0.001$) by 14% was detected only in Purkinje cells. The increase in neuronal soma volumes in nine regions in the teenagers/young adults and in six structures in the

older adults defined the dominant feature of the trajectory of neuronal volume changes during the lifespan of autistic subjects.

The pattern detected in autistic cohort is in strong contrast with opposite trend observed in the control cohort. In none of 16 examined regions did the volume of neuron soma increase in the 14- to 23- and in the 29- to 64-year-old control individuals, except a small (2%; $p < 0.018$) increase in the magnocellular basal complex of adults. Significant decrease of neuron soma volume was detected in the 14- to 23-year-old control subjects in seven regions, including Purkinje cells, claustrum, amygdala and magnocellular basal complex, -2%; thalamus, -15%; putamen, -19% and in the inferior olive, -18%, overall the neuron volumes decrease by 9%. In control individuals from 29 to 64 years old a further significant decrease of neuron soma volume was detected in five regions, including the nucleus accumbens, -9%; Purkinje cells, -5%; amygdala, -1%; caudate nucleus, 13%, putamen, -16% and in the Ammons horn, -3%, with a mean neuron volume reduction of 8%.

Discussion

Age of onset of the distortion of neuronal growth in autism

This postmortem study was designed to track developmental and age-associated modifications in the brain of autistic subjects. Neuronal soma volumes were selected as a technically, relatively precisely controlled marker of neuronal developmental and age-associated modifications both in the normal developing brain and in the brain with developmental abnormalities. The most severe neuronal volume deficits found in the 4- to 8-year-old children with autism suggest that a dysregulation of neuronal growth occurs before the age of four years. The significantly smaller volumes of neuronal soma in all 16 brain structures examined that are involved in all the diagnostic autism domains suggest that these early alterations contribute to the broad spectrum of clinical autism manifestations. The onset of the clinical features of autism is gradual in many children, but in other children, a functional regression has been reported in the first 1–2 years [49, 53, 83, 99]. Some studies suggest a prenatal onset of developmental abnormalities leading to autism [69, 71]. The pattern of changes seen in the cerebellum suggests that the pathology was acquired early in development [12]. This view is also supported by our findings of focal pre- and postnatal developmental defects, including abnormal migration with heterotopic and ectopic clusters of neurons, dysplastic changes, and subependymal nodular dysplasia detected in the brain of individuals with idiopathic and dup(15)- associated autism [130, 133].

Trajectory of neuronal growth in the brain of individuals with autism and trajectory of clinical changes.

The concept of age-associated changes of neuronal growth was explored in the first neuropathological studies of brains of subjects diagnosed before the introduction of the currently used diagnostic criteria for autism [12, 13, 66, 67]. A study of the brain of six individuals with autism 9, 10, 12, 22, 28, and 29 years of age revealed age-associated changes of neuronal size. In the younger autistic subjects (ages 9, 10, and 12 years), unusually large neurons were detected in some brain regions, including the vertical limb of the nucleus of the diagonal band of Broca and the fastigeal, emboliform, and globose nuclei in the cerebellum. In contrast, in the older subjects (22, 28, 29 years of age), the neurons in these same brain subdivisions were small and were reduced in number [14, 66]. The presence of large neurons in the younger subjects, and of small, closely packed neurons in the older subjects was interpreted as a sign of the curtailment of normal neuronal development [14]. The loss of skills over time by some individuals and the plateau in adolescence or the continued improvement in adulthood in other individuals reflect significant developmental heterogeneity [65, 126, 140]. Modest improvement in symptoms may be evident from childhood to adolescence, and from adolescence to adulthood. However, some individuals do not improve or reveal functional declines, especially very low- functioning individuals with severe symptoms and with seizures [94].

Reciprocal social interactions. The application of the ADI-R to detect changes of the pattern of autism symptoms in a large sample of individuals (n = 405) between the ages of 10 and 53 years has revealed that adolescents are more likely to improve in the reciprocal social interaction domain than are adults, whereas adults are more likely to improve in the restricted, repetitive behaviors and interests domain [116]. Mesibov et al.'s [88] study of the course of changes between childhood and adolescence suggested only limited improvements of social impairments. Improvements in the social domain in more than 80% of 38 high-functioning male and female adolescents and young adults have been reported by Piven et al. [101], although limitations in this domain remained for virtually all members of the examined cohort. Complex neuronal networks are involved in social cognition and emotional processes, including the amygdala, the cortex of the temporal superior gyrus and fusiform gyrus, medial prefrontal cortex, and anterior cingulate cortex [10, 91, 139]. Several studies have revealed amygdala alterations in individuals diagnosed with autism [11, 66, 112, 113]. Complementing these findings, our study of the trajectory of neuronal volume changes during the lifespan of individuals with autism reveals 20%, on average, neuronal volume deficits in the 4- to 8–year-old children, 15% deficits in teenagers/young adults and 9% deficits in adults, when compared to age-matched controls. Neurons in the amygdala and the Purkinje cells represent the neuronal populations with the most persistent and prominent volume deficits in teenagers and adults in comparison to 14 other brain regions. The limited correction of developmental deficits of neuronal volume in the amygdala may be a factor that limits improvement of the social impairments in autism during the lifespan.

Deficits in communication. Several retrospective and prospective longitudinal studies have documented improvements in communication from childhood to adolescence and adulthood [94]. Modest communicative improvements have been reported in a British sample [61, 86]. Speech improvement has been reported in a Japanese sample of 187 adults with autism between the ages of 18 and 33 years [70]. Seltzer et al.'s retrospective study of a community sample of more than 400 individuals divided into two age groups including adolescents (10–21 years) and adults (22 years or older, mean age = 31.6 years) revealed significant improvement in the adolescent cohort, and even more prominent improvement in the adult cohort [116]. Speech symptoms, considered "classic" signs of autism, including neologisms and pronominal reversal, improved the most, whereas limitations in pointing to express interest and use of gestures to communicate were less likely to improve [117]. Clinical impairments may be a reflection of structural changes in functionally related brain regions. The posterior inferior frontal cortex (Brodmann areas 44 and 45) is involved in language processing, including language production and syntactic processing in the left hemisphere [81, 129] and processing of the metaphoric meaning of sentences in the right hemisphere [17, 52]. Postmortem studies have revealed that the volume of the pyramidal neuronal soma was reduced in layers III, V, and VI in this cortex in individuals with autism [64]. The thalamus (especially the lateral thalamus) is involved in mechanical processing for articulation and respiration. In our study, thalamic neurons had very significant neuronal volume deficits in children with autism (-27%; $p < 0.001$), but neuronal volume increases were found to approach control levels in adolescents with autism and to exceed control levels in adults with autism (+9%; $p < 9\%$). Significant increases of neuronal size in such language-related structures as the thalamus and cerebellum may be structural correlates of communication improvements. Considerable variability in the course of change across communicative behaviors may reflect different levels of delayed neuronal growth in early childhood and different levels of neuronal volume correction in neuronal networks involved in communication and speech production in adolescents and adults.

Restricted, repetitive behaviors and interests. A key domain of the autism diagnostic criteria includes abnormal behavioral features such as stereotypes, obsessions, and insistence on sameness. Improvements in restricted and repetitive behaviors during the transition from childhood to adolescence and adulthood have been reported in prospective [88] and retrospective studies [116]. A study of 712 individuals diagnosed with autism spectrum disorder (ASD) from 2 to 62 years of age revealed that repetitive behaviors are a heterogeneous group of behaviors and that stereotyped movements and restricted interests exhibit the most pronounced age-related differences, whereas self-injurious behavior and compulsive behavior exhibit the least pronounced age-related differences [42]. Several studies implicate the role of basal ganglia and fronto-striatal circuitry in the autism phenotype, especially in repetitive and stereotyped behaviors. MRI studies have revealed an increased volume of basal ganglia [59, 60, 76, 114] and correlations between caudate nucleus volume and repetitive behavior scores [60, 114].

Our finding of a very severe neuronal volume deficit in the nucleus accumbens (-34%), and severe/moderate deficits in the caudate nucleus (-22%), globus pallidus (-20%), and putamen (-17%) in individuals with autism from 4 to 8 years of age may reflect the severity of restricted and repetitive behaviors in children with autism.

Daily living skills. A study of daily living skills, defined as behaviors necessary for age-appropriate, independent functioning in social, communication, or motor areas, in a large cohort of individuals with ASD (n = 397) that were evaluated over a 10-year period using the Waisman Activities of Daily Living Scale, revealed an improvement during adolescence and in the early 20s and plateau during the late 20s [120]. This improvement in a broad spectrum of daily leaving skills may reflect the trajectory of neuronal volume increases we found in all 16 examined structures. Daily leaving skills constitute a critical domain of adaptive behavior. The claustrum, which integrates multiple inputs from sensory cortical areas and redirects sensory information to subcortical structures, plays a unique role in the integration of different signals and adaptations to changing demands [41]. Our findings of a neuronal soma deficit in the claustrum by 29% in children, and a reduction of the neuronal volume deficit to 17% during adolescence and to 4% in adults suggest that age-associated corrections in the claustrum may play a significant role in the partial improvement of the adaptability and daily living skills of individuals with autism.

Unchanged or decreasing volume of neurons in control subjects

The increase in neuronal volumes to control levels in 10 brain regions in autistic subjects from 11 to 23-year-old, and in five regions significantly above control levels in autistic individuals from 36 to 60-year-old is in striking contrast with the absence of change or decrease with age of neuronal volumes we observed in 10 regions of age-matched control subjects. Similar decreases in perikaryal volume of neurons in layer V in the fusiform gyrus have been reported in control subjects from 4 to 65 years of age [130]. A report by Jacot-Descombes et al. [64] also demonstrated a decrease in pyramidal neuronal volumes in BA 44 and 45 across the lifespan of control but not autistic subjects. Andersen et al. [7] detected in a control cohort an age-associated reduction of the size of the Purkinje cell perikaryon. A reduction of the size of perikarya was also detected in the external pyramidal layer in the superior part of the precentral gyrus but only in 85- to 94-year-old subjects, and these changes were interpreted as a sign of age-associated neuronal atrophy [111]. It is not known why the volume of cortical pyramidal neurons, Purkinje cells, and neurons in subcortical structures decreases in control subjects, but the absence of this decline in autistic subjects in some structures and the increase in neuronal size in other brain regions appear to be a marker of the abnormal trajectory of neuronal growth in teenagers and adults with autism.

Global pattern of abnormal growth of neurons in the brain of autistic subjects and region-specific trajectories of neuronal growth

Small neurons in the neocortex of autistic individuals. Small sizes of neurons in autistic subjects have been reported in neuropathological evaluations without morphometric support [12, 13, 66, 67], and in morphometric studies that estimate neuronal volume [64, 108, 119, 130] or neuronal cross-sectional areas [43]. A small size of neurons has been reported in 22- to 29-year-old autistic subjects in the hippocampus, amygdala, medial septal nucleus, cerebellar nuclei, and inferior olive [12, 13, 66, 67]. Casanova et al. [26] reported reduced minicolumn widths, increased neuronal density, and reduced neuronal size in the superior and middle frontal gyrus in six autistic subjects 4–24 years of age compared to six age-matched control subjects. A study of seven autistic subjects, 4 to 23 years of age, and 10 control subjects, 4 to 65 years of age, revealed a reduced mean perikaryal volume of neurons in layers V by 21%, and VI by 13.4% in the fusiform gyrus. A reduced total neuronal number and smaller neurons in the main output layers of the fusiform gyrus were functionally linked to impaired face processing in autism [131]. A study of the inferior frontal cortex areas involved in language processing, imitative functioning, and social processing (Brodmann areas 44 and 45) revealed smaller pyramidal neuronal volumes in layers III (-18%), V (-18.5%), and VI (-22%) in seven autistic subjects, 4 to 52 years old, compared to seven control subjects, 4 to 48 years of age [64]. A study of the anterior cingulate cortex in nine males with autism from 15 to 54 years of age revealed significant decreases in neuronal volumes in layers I–III and V–VI [119], suggesting a contribution to modified affective and cognitive behaviors, socio-emotional attachments, emotional self control, adaptive responses to changing conditions, goal-directed behavior, joint attention, and motor activity [4, 135].

The majority of the brains of autistic subjects examined in four other morphometric studies [26, 64, 74, 130] represent a portion of the cohort examined in this study. The reduced volume of neurons detected in all 16 examined subcortical structures, archicortex, cerebellum, and inferior olive in the brainstem, combined with the results of the neocortical and brainstem studies of other investigators, strengthens the hypothesis of the global nature of defective neuronal growth being present in the brain of autistic subjects.

Amygdala, hippocampus, and entorhinal cortex. All individuals with autism display features of a deficit of social behavior, including abnormalities in social reciprocity and difficulties in the use of eye contact, facial expression, and social motivation [82, 103]. Neurobiological models of social cognition suggest that in the neuronal network engaged in social cognition, including the amygdala, the cortex of the temporal superior sulcus, and the fusiform gyrus, it is the amygdala that is responsible for labeling events with emotional meaning [10, 139]. Moreover, the amygdala plays a role in the detection of threats and the mobilization of an appropriate behavioral response including fear and anxiety [2].

Excessive anxiety is reportedly observed in 84% of children with autism [92]. Neuropathological data [66] as well as the results of structural [112, 121] and functional neuroimaging [11] provide evidence that the amygdala is affected in autism and that pathology of the amygdala may contribute to the clinical deficits of autistic subjects. Our observed 24% volume deficit of neurons ($p < 0.001$) in the amygdala of the 4- to 8-year-old autistic subjects in comparison to control children suggests that during the most critical stage of development of social behaviors and emotional relationships, the growth of neurons in the amygdala is altered. The observed persistent neuronal volume deficit of 10% ($p < 0.008$) and 13% ($p < 0.011$) in the amygdala of teenagers/young adults and in older adults, respectively, supports the concept of an ongoing abnormality of amygdala function. Smaller and more densely packed neurons were found in various portions of the hippocampal formation, the entorhinal cortex, and the medial nuclei of the amygdala [12, 66]. The finding of a reduced size of neurons to a comparable range in the entorhinal cortex (-24%; $p < 0.046$) and in the amygdala (-22%; $p < 0.001$) in the 4- to 8-year-old children with autism, but of only a 12% neuronal volume deficit ($p < 0.006$) in the Ammon's horn, suggests that the limbic system is developmentally affected, but the range of alterations in closely connected, and interacting structures may be different.

Claustrum. The claustrum receives inputs from many cortical areas, integrates multiple inputs into new signals, and redirects sensory information throughout the striatum and thalamus. Interconnectivity with subcortical nuclei and sensory cortical areas indicates the claustrum's involvement in sensorimotor integration and potentially the most complex human brain function, consciousness, as well as in higher orders of functionality, enabling the organism to rapidly adapt to the subtleties and nuances of a changing environment [41]. An attraction to routines and to sameness appears to be one of the striking behavioral alterations characteristic of autism. It appears that developmental alterations of the claustrum, indicated in the neuronal soma deficit of 29% in children, of 17% in teenagers/young adults, and of only 4% in older adults ($p < 0.001$), reflects claustral neurons' functional impairment contributing both to deficits of adaptability and consciousness and to a partially delayed reduction of the deficit with age similar to that seen in the limbic system.

The differences in the range of developmental delay of the claustral neurons and the neurons receiving claustral projections and projecting to the claustrum, suggest that claustral dysfunction is associated with or caused by desynchronized development of the subcomponents of these multifunctional networks. The claustrum is involved in long-term response potentiations within the claustral–entorhinal–hippocampal system [138], and we observed neuronal volume deficits of 29%, 24% and 12%, respectively. Neurons in the frontal, temporal, parietal, and occipital cortices project to the claustrum [39, 80], whereas neurons in the dorso-caudal claustrum (visual claustrum) project to the visual cortex. Only a slight reduction of neuronal volume in Brodmann area 17 in the occipital cortex [26] and a significant reduction in the mean perikaryal volume of the neurons in layers V and VI (by 21.1% and 13.4%,

respectively) in the fusiform gyrus [130] are indicative of a desynchronized development of the claustro-cortical networks in autism. It may affect the role of the claustrum as an integrator of input from the somatosensory, auditory, and visual cortices, as well as from the respective diencephalic relays [122]. The 12% and 5% deficits, respectively, in the neuronal volumes of the magnocellular and parvocellular lateral geniculate nucleus, and the 27% deficit in the neuronal volume in the thalamus may indicate a developmental desynchronization of claustrum-related regions. The rostral portion of the claustrum projects to the caudate nucleus [8], which is also affected by a 12% deficit in neuronal volume.

Magnocellular basal complex (MBC). The MBC consists of four major nuclei that send cholinergic, GABAergic, and glutamatergic axons to the cortical mantle, amygdale, and many subcortical structures [89, 109, 131], and its associated abnormalities may contribute to the clinical phenotype of autism. The cholinergic drive to the forebrain plays a modulatory role in anxiety, arousal, and attention and is essential for learning and memory tasks [68, 93]. The anteromedial part of the MBC (CH4) acts as the cholinergic relay for transmitting limbic and paralimbic information to the neocortex, thereby influencing complex behavior (integrated emotional and motor responses, learning, and memory), according to the prevailing emotional and motivational states encoded by the limbic and paralimbic brain structures. Ch4 neurons respond to the sight and taste of food, visual and auditory information. All the structures that project to the Ch4 are integrative regions of extensive sensory processing or regions of polysensory convergence. The 22% neuronal soma volume deficits observed in MBC in 4- to 8-year-old autistic subjects may reflect a defective function of the cholinergic system in early childhood. The reduced volume of neurons in the MBC of autistic subjects may result in an altered cholinergic innervation of the cortical mantle and contribute to anxiety, arousal, attention deficit, and learning difficulties. The outcome of the combination of (a) region/cell type–specific deficits of neuronal volumes and (b) systemic defects of cholinergic innervation may contribute to the broad spectrum of autistic phenotypes, including communication and social deficits, and repetitive and stereotyped behaviors.

Thalamus. Experimental studies have shown that almost all amygdaloid nuclei project to the thalamus and that their main target is the rostral half of the magnocellular mediodorsal thalamic nucleus [1, 106], which is involved in attention, emotional processing, anxiety, obsessive thinking, agitation, and assaultive behavior [95–97]. The lateral thalamus is closely related to language function, including the mechanical processes for articulation and respiration. The pallidal input to the thalamus serves to control muscle tone. MRI studies have revealed significantly reduced mean thalamic volume in high-functioning autistic subjects [128]. Our observations of much smaller neuronal soma (-27%; $p < 0.001$) in the thalamus of autistic subjects 4 to 8 years of age indicate that the neuronal circuits involved in articulation, attention, anxiety, and obsessive thinking belong to the most developmentally affected brain structures. The increase in neuronal soma volumes to control levels in teenagers/young adults and to significantly

above control level in older adults (9%; $p < 0.007$) may reflect age-associated modifications of neuronal growth and possibly function.

Cerebellar cortex, dentate nucleus, and inferior olive. A reduced number and size of Purkinje cells was observed in the majority of cerebellar analyses in 21 of 29 examined cases [100]. Quantitative studies suggest that a regional decrease in the number of Purkinje cells may be the result of a prenatal loss of Purkinje cells [136, 137]. Fatemi et al. [43] proposed that the 24% reduction in the cross-sectional areas of Purkinje cells that they detected in five young adults with autism (25 years of age on average) was a sign of neuronal atrophy. Our study revealed very severe Purkinje cell soma volume deficits (-31 %; $p < 0.001$) in 4- to 8-year-old children with autism, severe in 11- to 32-year-old (-15%; $p < 0.008$) and moderate (-23%; $p < 0.001$) in subjects more than 29-year of age. These signs of developmental alterations coincide with a 40% decrease in the expression of glutamic acid decarboxylase 67 (GAD67) mRNA in autistic subjects [141] and an increased GABAergic feed-forward inhibition to Purkinje cells by basket cells, suggesting a disruption in the timing of Purkinje cell firing and altered inhibition of the cerebellar nuclei, which could directly affect cerebellocortical output, leading to changes in motor behavior and cognition [142]. Experimental studies demonstrate that Purkinje cell activity is controlled by the inferior olive climbing fibers [14, 87]. Maturation of cerebellar connectivity and motor activity are associated with a transition from multiple climbing fibers' innervation of Purkinje cells to a single climbing fiber innervation to each Purkinje cell [27]. A common excitatory input from the climbing fibers of a distinct subgroup of the inferior olive neurons to Purkinje cells defines the distinct subcompartments (microzones) of the cerebellar cortex [63, 98]. Our observed smaller volumes of Purkinje cells by 31%, of the dentate nucleus by 25%, and of the inferior olive by 14% in 4- to 8-year-old autistic subjects ($p < 0.001$) compared to age-matched controls and the different age-associated trajectory of neuronal growth in older autistic subjects, with increases of neuronal volume only in the inferior olive, suggest that different components of the brainstem-cerebellar circuits are affected by developmental alterations and that the range of age-associated neuronal volume modifications is brain region–specific. A unique form of developmental disruption of brainstem/cerebellar networks is floccular dysplasia, which may contribute to the oculomotor system alterations resulting in avoidance of eye contact and poor or no eye contact observed in autistic subjects [134].

The small size of the neurons in the inferior olive is not an isolated sign of developmental abnormalities in the brainstem. A study of the auditory system in the brainstem of autistic subjects revealed a reduced size of the neuronal soma in the medial superior olive [73] as well as neuronal deficits and reduced neuronal size in the superior olivary complex that may contribute to the auditory deficits observed in autistic patients [74].

Nigro-striatal system. The substantia nigra, caudate nucleus, putamen, nucleus accumbens, and globus pallidus belong to the basal ganglia. They form cortico-subcortical circuits that play a key role in

programming and execution of movements and in the reward system. The reward system includes (a) the prefrontal cortex, (b) the limbic system components, including the amygdala, hippocampus, and anterior cingulate gyrus, and (c) the substantia nigra and limbic striatum, including the nucleus accumbens, ventral caudate nucleus, and putamen [18, 28, 36, 90]. The autism phenotype includes lower-order repetitive motor behaviors, intense circumscribed patterns of interests, and higher-order rituals and compulsions that occur regularly and interfere with daily functioning [46]. An inverse correlation between the caudate nucleus volume and the presence of compulsions and rituals, and difficulty in changing routines was detected in 12- to 29-year-old autistic patients [114]. An increased volume of the caudate nuclei was proportional to compulsions and rituals [115]. The volume of the right caudate nucleus correlates with repetitive behaviors in 17- to 55-year-old ASD subjects [60]. Because of its anatomy and connectivity, the nucleus accumbens is considered a mixed structure with elements of the striatum and "extended amygdala" [57]. The nucleus accumbens is required for a number of reward-related behaviors, and it processes specific information about reward availability, value, and context [36]. Its projections to motor areas such as the ventral pallidum turn reward information into motivated action [90]. This study revealed significantly smaller ($p < 0.001$) volumes of neurons in the substantia nigra (-5%), caudate nucleus (-12%), putamen (-17%), globus pallidus (-20%), and nucleus accumbens (-34%) of children with autism. These findings suggest that repetitive motor behaviors, circumscribed patterns of interests, rituals, and compulsions are the functional consequences of the abnormal development and maturation of nigro-striatal networks. However, in teenagers and adults with autism, all these structures revealed an increase of neuron soma volume to or significantly above (substantia nigra; +19%; $p < 0.001$) control level. The striking shift in neuronal volume from deficit in children with autism to control level in teenagers/adults with autism but persistent ritualistic behaviors may indicate that delayed neuronal growth does not restore functional connectivity and function, or corrections are very limited.

Small size of neurons in other disorders. A smaller than normal size of neurons has been reported also in schizophrenia and has been linked to the prevailing hypothesis that schizophrenia is a disorder of prenatal neurodevelopment [54]. The smaller mean size of hippocampal pyramidal neurons [9, 15, 143] and neurons in the prefrontal cortex [102] and of Purkinje cells in the cerebellar vermis [127] suggests that defects of the growth of neurons in schizophrenia are limited to only a few brain regions that are also affected in autism. A 17–20% smaller mean neuronal soma size in the Ammon's horn [125], in the dorsolateral prefrontal cortex (BA 9) [30], and in the anterior cingulate gyrus [29] reported in individuals with depression suggests disorder-specific patterns of neuronal growth alterations.

Closing remarks. Recent genetic findings along with anatomical and functional imaging studies suggest an ASD model in which higher-order association areas of the brain, which normally connect to the frontal lobe, are partially disconnected during development ("a developmental disconnection syndrome") [33, 45, 48]. According to Geschwind and Levitt [48], the disconnection in ASDs is not

primarily a disruption of previously connected regions but rather a failure of them to develop normally. Our results of the morphometric studies of 16 brain regions and the results of other neocortical studies demonstrate a failure of normal neuronal development that may contribute to a failure of normal connectivity development. Estimates of brain region–specific neuronal volume deficits and different trajectories of neuronal volume modifications during the lifespan indicate that an integral component of this developmental failure is the desynchronized growth of neurons in children, teenagers, and adults with autism in neuronal networks, and that this is a major structural contribution to the autism phenotype. The trajectory of neuronal growth detected in individuals with autism 4–8, 11–23, and 36–60 years of age resulting in an approximation of neuronal soma volumes close to those of control adults appears to reflect delayed up-regulation of neuron growth with limited contribution to partial improvement in all three diagnostic autism domains and daily living skills during the transition from childhood to adolescence, from adolescence to adulthood, and during adulthood [116–118, 120]. The disproportion between (a) a strikingly strong trend to increase neuron soma volume close to control level in majority of examined brain regions in autistic teenagers and adults, and (b) limited clinical improvement during autistic individuals lifespan suggests that mechanisms controlling delayed neuron growth do not replicate normal neuron development, maturation and connectivity.

Due to significant developmental heterogeneity, clinical studies of developmental trajectories and age-associated modifications in autism symptoms have required the evaluation of 241 to 700 individuals with ASD [42, 116, 118, 120]. The size of postmortem cohorts examined allows defining of brain region–specific trajectories of neuronal growth, but studies of correlations between morphometric markers of neuronal developmental changes during the lifespan and age-associated modifications of the pattern of clinical changes may require much larger samples.

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Table 1 Autism diagnosis, prevalence of seizures, and SUDEP

Case number	Sex	Age (years)	Autism Diagnostic Interview- Revised (ADI-R)				Seizures age of onset and SUDEP
			Social deficits (10)	Communication deficits		Repetitive behavior (3)	
				Verbal (8)	Nonverbal (7)		
1	M	4	14	NA	10	3	—
2	F	5	26	NA	11	4	—
3	M	7	29	NA	14	3	14 months
4	M	8	19	14	NA	4	—
5	F	11	22	14	NA	3	4.5 months SUDEP
6	M	13	28	NA	12	3	2 years SUDEP
7	F	17	15	16	NA	7	—
8	F	21	21	NA	11	7	5 years SUDEP
9	M	22	28	NA	14	6	15 years SUDEP
10	M	23	30	NA	14	8	23 years SUDEP
11	M	36	23	NA	10	6	—
12							—
13	M	56	19	8	13	3	—
14	M	60	26	8	14	4	3 years

ADI-R cut-off score shown in parentheses.

SUDEP, unexpected and unexplained death that occurs in patients with known epilepsy.

Table 2 Tissue samples, demographics of autistic and control subjects, brain weight, and changes during processing

Group	Case number	Sex	Age (years)	PMI (h)	Brain weight (g)	H	Fixation (days)	Dehydration (days)	Weight loss (%)
A	1	M	4	30	1,280	R	4,560	28	49
A	2	F	5	13.2	1,390	R	1,568	28	52
A	3	M	7	25	1,610	R	330	37	55
A	4	M	8	22.2	1,570	R	196	36	45
A	5	F	11	12.9	1,460	L	308	34	52
A	6	M	13	8	1,470	L	75	33	39
A	7	F	17	25	1,580	L	470	36	51
A	8	F	21	50	1,108	R	136	35	43
A	9	M	22	25	1,375	R	1,034	39	38
A	10	M	23	14	1,610	R	533	45	60
A	11	M	36	24	1,480	R	721	37	44
A	12	M	52	27.6	1,625	R	1,650	39	40
A	13	M	56	3.3	1,570	R	692	38	52
A	14	M	60	26.5	1,210	R	398	39	38
Average				**21.9**	**1,453**		**905**	**36**	**47**
SD				**11.4**	**162**		**1,160**	**4**	**7**
C	1	F	4	17	1,530	R	126	41	49
C	2	F	4	21	1,222	R	233	30	43
C	3	M	7	12	1,240	R	130	41	51
C	4	F	8	20	1,222	R	650	36	51
C	5	M	14	20	1,464	R	1,067	38	44
C	6	F	15	9	1,250	R	372	41	49
C	7	F	20	9	1,340	R	245	37	52
C	8	M	23	6	1,520	R	95	45	41
C	9	M	29	13	1,514	R	89	41	49
C	10	M	32	24	1,364	R	460	37	42
C	11	M	48	24	1,412	L	215	38	39
C	12	M	51	18	1,450	L	1,819	20	26
C	13	M	52	13	1,430	L	158	47	48
C	14	M	64	28	1,250	R	52	37	51
Average				**16.7**	**1,372**		**408**	**38**	**45**
SD				**6.6**	**118**		**491**	**7**	**7**

PMI, postmortem interval; h, hours; H, hemisphere; R, right; L, left; SD, standard deviation.
Fixation: duration (in days) of fixation in 10% buffered formalin.
Dehydration: duration (in days) of dehydration in ethyl alcohol.
Weight loss: decrease of hemispheric brain sample weight during dehydration in ethyl alcohol.

Table 3 Parameters and procedures applied to estimate the volume of neuronal soma

Structures and their subdivisions	Number of equal distance sections examined (per case)	Obj.	Grid size (µm)	Test area frame x height (µm)	Mean number of virtual counting spaces (per case)	Mean number of neurons measured (per case)	CE*
Amygdala	12	40x	1000x1000	80x80x10	356	454	0.01
Thalamus	6	40x	1000x1000	80x80x10	188	313	0.01
Entorhinal c. LII	6	40x	400x400	60x60x30	109	117	0.003
LIII			800x800	60x60x30	117	139	
LV			600x600	60x60x30	62	122	
LVI			800x800	60x60x30	67	124	
CA1	14	40x	1000x1000	60x60x30	53	139	0.003
CA2			400x400	60x60x30	66	122	
CA3			400x400	60x60x30	79	118	
CA4			600x600	100x100x30	34	130	
Caudate nucleus	4	63x	2000x2000	80x80x30	74	244	0.002
Putamen	4	63x	2000x2000	80x80x30	73	255	
Globus pallidus	4	63x	500x500	180x180x30	117	137	
Nucleus accumbens	4	63x	500x500	180x180x30	134	353	
Magnocellular LGB	4	40x	500x500	100x100x30	114	190	0.002
Parvocellular LGB	4	40x	500x500	100x100x30	123	211	0.002
Claustrum	9	40x	250x250	60x60x10	274	318	0.01
Substantia nigra	9	63x	300x300	80x80x30	108	287	0.002
Magnocellular basal complex (Ch1-Ch4)	9	63x	300x300	80x80x30	59	153	0.005
Purkinje cells	4	40x	1800x1800	180x180x30	664	253	0.002
Dentate nucleus	4	40x	1000x1000	180x180x30	145	254	0.002
Inferior olive	4	40x	1000x1000	180x180x30	132	260	0.002

Obj., objective; *CE, the average predicted coefficient of error of the measured neurons (Schaffer); LGB, lateral geniculate body.

Table 4 The difference between the mean volume of neuronal soma in autistic and control cohorts

Brain structure	4- to 8-year-old subjects		11- to 23-year-old subjects		29- to 64-year-old subjects	
	Control	Autism	Control	Autism	Control	Autism
	Mean (LSE) (100%)	Mean (LSE) *p* < (%)	Mean (LSE) (100%)	Mean (LSE) *p* < (%)	Mean (LSE) (100%)	Mean (LSE) *p* < (%)
Nucleus accumbens	1,181 (9)	778 (7) 0.001 **-34%**	1,090 (8)	1,021 (7) 0.242 ns	997 (7)	1,102 (8) 0.366 ns
Cerebellum. Purkinje cells	11,635 (104)	8,047 (97) 0.002 **-31%**	11,460 (112)	9,774 (80) 0.008 **-15%**	10,845 (82)	8,389 (85) 0.001 **-23%**
Claustrum	1,994 (14)	1,407 (13) 0.001 **-29%**	1,950 (14)	1,614 (11) 0.001 **-17%**	1,785 (13)	1,722 (19) 0.001 **-4%**
Thalamus	3,702 (36)	2,682 (35) 0.001 **-27%**	3,135 (37)	3,051 (30) 0.388 ns	3,230 (31)	3,535 (39) 0.007 **+9%**
Dentate nucleus	8,243 (153)	6,218 (121) 0.010 **-25%**	8,331 (158)	7,106 (103) 0.017 **-15%**	7,732 (112)	7,006 (124) 0.209 ns
Amygdala	3,033 (28)	2,309 (26) 0.001 **-24%**	2,982 (30)	2,674 (22) 0.008 **-10%**	2,946 (27)	2,552 (27) 0.011 **-13%**
Entorhinal cortex	2,424 (29)	1,841 (23) 0.046 **-24%**	2,450 (37)	2,041 (23) 0.001 **-17%**	2,420 (23)	2,464 (27) 0.001 **+2%**
Magnocellular basal complex	8,385 (67)	6,553 (56) 0.001 **-22%**	8,214 (74)	8,063 (66) 0.650 ns	8,425 (83)	8,174 (126) 0.001 **-3%**
Caudate nucleus	1,199 (13)	936 (12) 0.001 **-22%**	1,218 (12)	1,192 (10) 0.069 ns	1,056 (10)	1,217 (11) 0.642 ns
Globus pallidus	5,636 (70)	4,502 (74) 0.001 **-20%**	4,478 (70)	4,354 (52) 0.325 ns	5,001 (56)	4,930 (59) 0.162 ns
Putamen	1,316 (12)	1,095 (12) 0.001 **-17%**	1,065 (11)	988 (8) 0.147 ns	891 (8)	993 (9) 0.746 ns
Inferior olive	4,465 (76)	3,833 (54) 0.001 **-14%**	3,661 (63)	4,172 (43) 0.127 ns	3,917 (42)	4,219 (85) 0.011 **+8%**
Ammon's horn	3,414 (31)	3,020 (28) 0.006 **-12%**	3,675 (32)	3,147 (25) 0.007 **-14%**	3,575 (27)	3,459 (29) 0.001 **-3%**
Magnocellular LGB	5,448 (77)	4,795 (68) 0.001 **-12%**	5,908 (87)	5,622 (64) 0.273 ns	6,046 (75)	6,197 (77) 0.001 **+2%**
Parvocellular LGB	2,605 (31)	2,466 (30) 0.001 **-5%**	2,828 (34)	2,524 (25) 0.081 ns	2,831 (30)	2,949 (31) 0.074 ns
Substantia nigra	9,008 (65)	8,604 (61) 0.001 **-5%**	8,957 (65)	9,087 (51) 0.077 ns	9,324 (71)	11,101 (116) 0.001 **+19%**

LSE, linearized standard error; n, LGB, lateral geniculate body. Significance levels computed controlling for post-mortem interval, days of fixation, days of dehydration, and weight loss during dehydration.

Table 5 The trajectory of neuronal volume changes during lifespan of autistic and control subjects

Brain structure	**Autism**					**Control**				
	A	**B**	**C**	**Diff %**	**Diff %**	**A**	**B**	**C**	**Diff %**	**Diff %**
	4-8 y	**11-23 y**	**36-60y**	**A/B**	**B/C**	**4-8 y**	**14-23 y**	**29-64 y**	**A/B**	**B/C**
Nucleus accumbens	779	1,021 0.001	1,102 0.564	**31%**	**8%** **ns**	1,181	1,090 0.372	997 0.001	**- 8%** **ns**	**-9%**
Cerebellum. Purkinje cells	8,047	9,774 0.247	8,389 0.001	**21%** **ns**	**-14%**	11,635	11,460 0.007	10,845 0.001	**-2%**	**-5%**
Claustrum	1,407	1,614 0.001	1,722 0.790	**15%**	**7%** **ns**	1,994	1,950 0.001	1,785 0.321	**-2%**	**-8%** **ns**
Thalamus	2,682	3,051 0.003	3,535 0.016	**14%**	**16%**	3,702	3,135 0.004	3,230 0.198	**-15%**	**3%** **ns**
Dentate nucleus	6,218	7,106 0.756	7,006 0.319	**14%** **ns**	**-4%** **ns**	8,243	8,331 0.732	7,732 0.105	**1%** **ns**	**-7%** **ns**
Amygdala	2,309	2,674 0.001	2,552 0.135	**16%**	**-5%** **ns**	3,033	2,982 0.001	2,946 0.015	**-2%**	**-1%**
Entorhinal cortex	1,841	2,041 0.002	2,464 0.001	**11%**	**21%**	2,424	2,450 0.865	2,420 0.080	**1%** **ns**	**-1%** **ns**
Magnocellular basal complex	6,553	8,063 0.001	8,174 0.274	**23%**	**1%** **ns**	8,385	8,214 0.001	8,425 0.018	**-2%**	**2%**
Caudate nucleus	936	1,192 0.001	1,217 0.001	**27%**	**2%**	1,199	1,218 0.528	1,056 0.001	**2%** **ns**	**-13%**
Globus pallidus	4,502	4,354 0.331	4,930 0.045	**-3%** **ns**	**13%** **ns**	5,636	4,478 0.267	5,001 0.084	**-21%** **ns**	**12%** **ns**
Putamen	1,095	988 0.028	993 0.026	**-10%** **ns**	**0%**	1,316	1,065 0.001	891 0.001	**-19%**	**-16%**
Inferior olive	3,833	4,172 0.001	4,219 0.221	**9%**	**1%** **ns**	4,465	3,661 0.001	3,917 0.098	**-18%**	**7%** **ns**
Ammon's horn	3,020	3,147 0.161	3,459 0.001	**4%** **ns**	**10%**	3,414	3,675 0.056	3,575 0.027	**8%** **ns**	**-3%**
Magnocellular LGB	4,795	5,622 0.020	6,917 0.061	**17%**	**23%** **ns**	5,448	5,908 0.865	6,046 0.933	**8%** **ns**	**2%** **ns**
Parvocellular LGB	2,466	2,524 0.173	2,949 0.065	**2%** **ns**	**17%** **ns**	2,605	2,828 0.205	2,831 0.617	**9%** **ns**	**0%** **ns**
Substantia nigra	8,604	9,087 0.202	11,101 0.001	**6%** **ns**	**22%**	9,008	8,957 0.646	9,324 0.883	**-1%** **ns**	**4%** **ns**

Significance levels computed controlling for post-mortem interval, days of fixation, days of dehydration, and weight loss. Results marked "ns" were non-significant using a False Discovery Rate (FDR) of 0.05.

Figures

Fig. 1 Smaller size of neurons of autistic subject 8 years of age (A) in comparison to age-matched control subject (C) shown in order from the largest to the smallest neurons: Purkinje cells (Pc), neurons in the magnocellular basal complex (MBC), dentate nucleus (DN), globus pallidus (GP), inferior olive (IO), thalamus (Th), amygdala (Am), in the island in the second layer of the entorhinal cortex (EC), claustrum (Cl) and nucleus accumbens (Ac). Calibration bar, 20 μm.

Fig. 2 The graph illustrates the range of the mean volume of neuronal soma in 16 examined brain structures from the largest Purkinje cells (Pc) to the smallest neurons in the nucleus accumbens (Ac) in from 4- to 8-year old control subjects. In all 16 examined structures, in 4- to 8-year-old autistic subjects, the neuronal soma volume was significantly less ($p < 0.05$) than in age-matched control subjects, but the volume deficit (control, 100%) varied in examined regions and ranged from mild (< 10%), moderate (11–20%), severe (21–30%) to very severe (> 30%). Whiskers, SE. Symbols: Purkinje cells (Pc), substantia nigra (SN), magnocellular basal complex (MC), dentate nucleus (DN), globus pallidus (GP), magnocellular LGB (mL), inferior olive (IO), thalamus (Th), Ammons horn (AH), amygdala (Am), parvocellular LGB (pL), entorhinal cortex (EC), claustrum (Cl), putamen (Pu), caudate nucleus (CN), and nucleus accumbens (NA).

Fig. 3 Neuronal soma volume distribution in autistic (red line) and control (green line) subjects in brain structures with the largest neurons (Purkinje cells and neurons in the magnocellular basal complex), moderate size neurons (thalamus and amygdala), and small neurons (caudate nucleus and nucleus accumbens). Graphs show a significant shift towards small size in 4- to 8-year-old autistic subjects in all three neuronal size categories and a rather uniform reduction of differences in volume distribution in 11- to 60-year-old autistic subjects.

Fig. 4 Graphs demonstrate changes of the mean neuronal volume in three age groups of autistic and control individuals. In from 4- to 8-year-old autistic subjects, the mean neuronal soma volume is significantly less ($p < 0.05$) in all 16 examined brain regions (marked with black arrows) than in age-matched controls. The number of regions with significant deficit of neuronal soma volume decreases to six in autistic teenagers/young adults and to five regions in 36 to 60 years-old autistic individuals. In five structures of autistic individuals from 36- to 60-years-old neuronal soma volume significantly exceeds control level (marked with white arrows).

Fig. 5 Striking difference between trajectory of neuronal volume changes within autistic and control cohorts. Neuronal soma volume increases in 11 regions in autistic teenagers/young adults and older adults. Large white arrow marks structures with significant neuronal volume increase in both older groups. Small white arrow marks structures with significant volume increase in only one of two older groups. The opposite trend is observed in the control group: the volume of neurons decreases in 10

regions. Three large black arrows mark a significant decrease of neuron volume in both older groups. Seven small black arrows mark a significant decrease in one of two older groups.

Fig.1

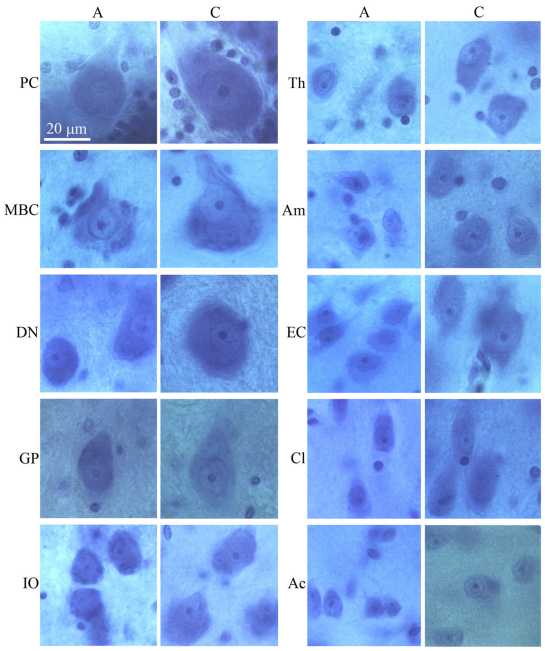


Fig. 2

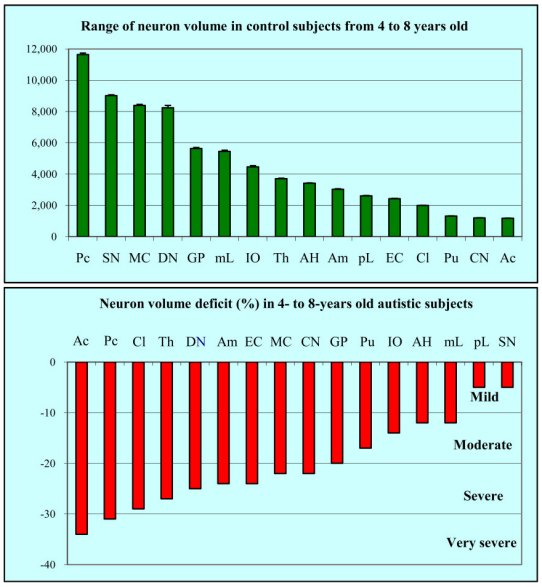

Range of neuron volume in control subjects from 4 to 8 years old
12,000
10,000
8,000
6,000
4,000
2,000
0
Pc SN MC DN GP mL IO Th AH Am pL EC Cl Pu CN Ac
Neuron volume deficit (%) in 4- to 8-years old autistic subjects
Ac Pc Cl Th DN Am EC MC CN GP Pu IO AH mL pL SN
0
-10
-20
-30
-40
Mild
Moderate
Severe
Very severe

Fig. 3

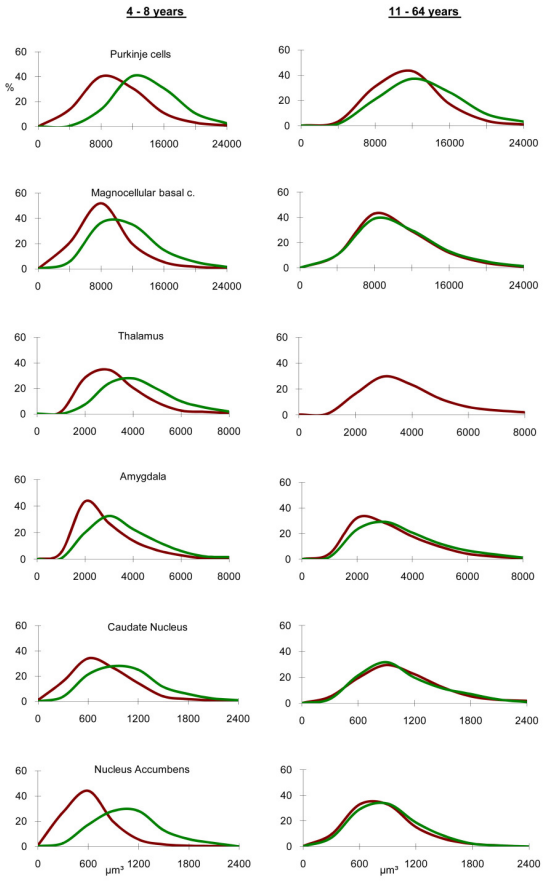

4 - 8 years
11 - 64 years
Purkinje cells
Magnocellular basal c.
Thalamus
Amygdala
Caudate Nucleus
Nucleus Accumbens
%
µm³
µm³

Fig. 4

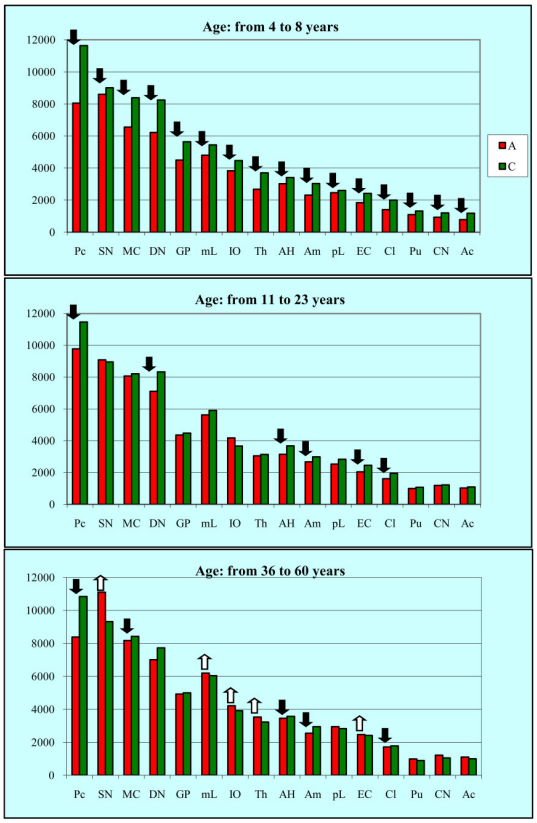
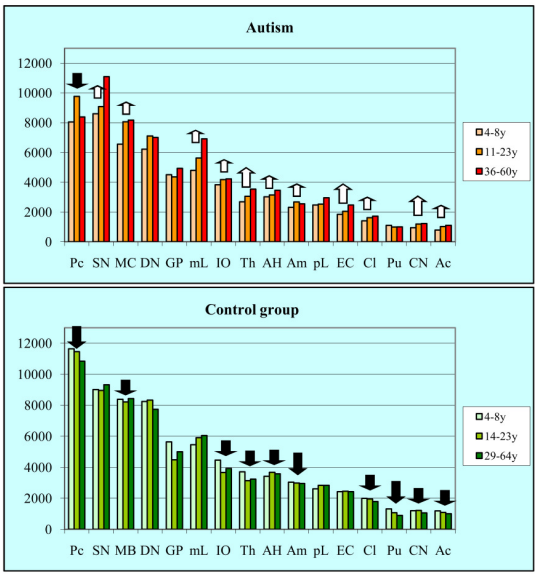

Age: from 4 to 8 years
12000
10000
8000
6000
4000
2000
0
Pc SN MC DN GP mL IO Th AH Am pL EC Cl Pu CN Ac
A
C
Age: from 11 to 23 years
12000
10000
8000
6000
4000
2000
0
Pc SN MC DN GP mL IO Th AH Am pL EC Cl Pu CN Ac
Age: from 36 to 60 years
12000
10000
8000
6000
4000
2000
0
Pc SN MC DN GP mL IO Th AH Am pL EC Cl Pu CN Ac

Fig. 5


Autism
12000
10000
8000
6000
4000
2000
0
Pc SN MC DN GP mL IO Th AH Am pL EC Cl Pu CN Ac
4-8y
11-23y
36-60y
Control group
12000
10000
8000
6000
4000
2000
0
Pc SN MB DN GP mL IO Th AH Am pL EC Cl Pu CN Ac
4-8y
14-23y
29-64y

Lower numerical density of neurons in only three of 30 examined cytoarchitectonic subdivisions in the brain of autistic individuals

Abbreviated Article Title: Number of neurons in autism

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Keywords Autism – Neuropathology - Brain - Number of neurons - Fractionator

Abstract.

Our prior postmortem studies of the brain of 14 autistic individuals 4 to 60-years-of age and 14 age-matched controls showed that (a) in 92% of autistic subjects at least one of three types of focal developmental defects is observed including abnormal neuronal migration, defects of neuronal proliferation and dysplasia, and (b) in all 16 examined brain regions the mean volume of neuronal soma is significantly less in 4- to 8-year-old autistic children than in control group but the size of neurons in majority of examined structures increases in adolescence and in adults to or above control level. The aim of this study of the same cohorts was to establish whether striking modifications of neuronal growth trajectories are associated with alterations of the volume of affected structures, the numerical density and the total number of neurons in these structures. In contrast to the surprisingly uniform pattern of altered neuronal growth, Cavalieri method revealed smaller volume of only one structure (n. accumbens) among 15 structures and their 21 subdivisions. Moreover, the estimates of the numerical density of neurons in 14-brain regions and their 16-cytoarchitectonic subdivisions revealed significantly lower density only in the amygdala lateral nucleus, putamen and n. accumbens of autistic subjects, whereas the fractionator revealed a lower total number of neurons only in islands in the entorhinal cortex. This stereological study of historically the largest cohort of autistic subjects and the broadest spectrum of structures, suggests a limited contribution of developmental alterations of the volume of brain structures and number of neurons to the autistic phenotype, and supports a key role of abnormal trajectory of neuron growth to behavioral changes in autism.

Introduction

Autism is a lifelong disorder characterized by disrupted development of social and communication skills, and restricted, repetitive and stereotypical patterns of behavior, interests and activities. In nearly 50 % of subjects later diagnosed with autism functional alterations are noted between 14 and 24 months, and at age of 24 months, all three diagnostic functional domains are affected [Lord et al 2000, DSMV]. In contrast to numerous clinical studies of large representative cohorts, the postmortem neuropathological studies are few, limited to evaluation of one to nine brains, and often restricted to a few brain regions. Different methods of tissue preservation and evaluation, and concentration on different aspects of brain pathology result in an inconsistent view of brain developmental defects and their contribution to clinical autism manifestations.

This project was designed to overcome some of these limitations and concentrate on the global pattern of developmental abnormalities rather than on localized models of brain pathology. To achieve this goal, almost twice many brains were examined (14 autistic and 14 age-matched controls) than in majority of previous reports. To establish a global pattern of changes this volumetric study was performed in 36 brain structures/cytoarchitectonic subdivisions, and neuronal counts were performed in 30 structures/subdivisions. Unbiased stereological studies of several cortical regions in a part of this cohort [van Kooten et al 2008, Santos et al 2011, Jacot Descomb et al 2012] expand the global view of developmental alterations in the brain in idiopathic autism.

Neuropathological study of this cohort revealed that in 92% of cases with autism at least one of three types of focal developmental abnormalities is observed, including defects of neuronal migration resulting in heterotopias, defects of neuronal proliferation with subependymal nodular dysplasia, and dysplastic changes in the cortex and subcortical structures. Observed types, topography and severity of alterations suggest that they may contribute to enhanced prevalence of seizures and SUDEP in autism [Wegiel et al 2010, 2012]. Application of Nucleator revealed that idiopathic autism is associated with a significantly smaller volume of neuronal soma in all 16 examined regions in the 4- to 8-year-old autistic children. A very severe neuronal volume deficit in 12.5% of examined structures, severe in 44%, moderate in 31%, and mild in 12.5% of structures reflects desynchronized neuronal growth in interacting neuronal networks. Altered trajectories of neuron growth with decrease in the number of brain structures with significantly smaller neurons from 16 in the 4- to 8-year-old autistic subjects to 6 in the 11- to 23-year-olds-, and to 5 in the 36- to 60-year-olds reflect delayed up-regulation of neuronal

growth during adolescence and adulthood reducing neuron soma volume deficit. Significant increase of neuron size but limited clinical improvement suggests that delayed correction does not fully normalize neuron structure and function [Wegiel et al 2013b].

One of the major arguments justifying concentration of this research on the volume of brain structures and number of neurons is a rapid increase in head circumference at the age of 1 to 2 years [Courchesne et al 2003, Dawson et al 2007, Dementieva et al 2005, Dissanayake et al 2006, Fombone et al 1999] coinciding with behavioral worsening and slowing of development in the second year [Landa and Garrett-Mayer 2006]. Courchesne et al [2001] revealed normal head size at birth but larger than normal head size in 90 % of 2- and 3-year-old children with autism. However, a slower rate of brain growth between 2 and 4 years of age [Carper et al 2002, Courchesne et al 2001, Hazlett et al 2005, Sparks et al 2002] results in only a 2 % brain overgrowth in autistic adults [Redcay and Courchesne 2005] or smaller brain size in comparison to control subjects [Hadjikhani et al 2006, Kosaka et al 2010]. The link between the deviation from the normal trajectory of brain growth and the severity of disease suggests a contribution of these changes to the clinical phenotype [Courchesne et al 2003]. Overgrowth of the frontal and temporal lobes and the amygdala but not the occipital lobe parallels brain overgrowth in 2- to 4-year-old autistic children and suggests that alterations are brain structure-specific. Accelerated growth in brain regions involved in cognitive, social and emotional functions and language development suggests that these structural alterations contribute to functional deficits [Carper et al 2002, Carper and Courchesne 2005, Courchesne et al 2001, Hazlett et al 2005, Sparks et al 2002]. However, the only direct evidence of an increased number of neurons in autistic subjects is the Courchesne et al report [2011] demonstrating 67% more neurons in the prefrontal cortex in autistic 2 to 16 year-old autistic subjects, and Santos et al (2011) report demonstrating 53% more von Economo neurons in the frontoinsular cortex in 4 to 11-year-old autistic individuals than in controls. On the other hand, several studies indicate that in an autistic individual the number of neurons in some regions is unmodified [Guerin et al 1996, Fatemi et al 2002, Kennedy et al 2007, van Kooten et al 2008, Santos et al 2011, Jacot Descomb et al 2012, Whitney et al 2009] or reduced [Ritvo et al 1986, Kemper and Bauman 1998, Simms et al 2009, Schumann and Amaral 2006, Kulesza et al 2011]. Therefore the specific aim of this study is to determine whether the detected brain-region or structure-specific modifications of the trajectory of neuron growth are associated with changes in the (a) volume of anatomical brain subdivisions, (b) numerical density and (c) total number of neurons per region of interest (ROI) in individuals diagnosed with idiopathic autism. We assume that increase of the number of examined cases and

number of examined brain regions, and application of one standard of stereological estimation, will help to reveal global pattern of developmental alterations in idiopathic autism.

Material and methods

Cohort exclusion criteria. Originally 39 brains were assigned for the study of several aspects of developmental abnormalities in autism, including 21 brains of autistic subjects and 18 control brains. Application of clinical inclusion criteria and neuropathological exclusion criteria reduced the size of this cohort to 28 subjects, including 14 autistic and 14 age-matched control individuals. Two cases did not meet the Autism Diagnostic Interview-Revised (ADI-R) [Lord et al 2004] criteria for diagnosis of autism. Due to severe autolysis one brain of autistic subjects and four control brains were excluded. Three brains of autistic subjects were excluded due to hypoxic encephalopathy and one due to multiple microinfarcts (Wegiel et al 2013b).

Autism diagnosis and clinical characteristics. Clinical diagnosis of autism was confirmed by postmortem application of ADI-R. Results of ADI-R application and clinical characteristics of this cohort have been summarized by Wegiel et al [2013b]. Eight subjects (61 %) were affected by mild to severe intellectual deficits detected using the Wechsler Intelligence Scale for Children III and the Woodcock-Johnson Tests of Achievement-Revised. Seven of 14 autistic subjects were diagnosed with seizures (50%) and in five cases (36%) the death was seizure-related.

Neuropathological evaluation of developmental alterations. The results of the study of developmental abnormalities summarized in our previous report [Wegiel et al 2012] revealed several types of developmental abnormalities in 13 brains of autistic subjects (92%), including (a) defects of proliferation with subependymal nodular dysplasia, (b) defects of migrations with subcortical and periventricular heterotopias and (c) dysplastic changes in the neocortex, archicortex, dentate gyrus, Ammons horn and cerebellum.

Tissue processing, embedding, sectioning and staining. Tissue samples, demographics of autistic and control subjects, brain weight and changes during processing are shown in Table 1 and characterized in detail in previous report [Wegiel et al 2013b]. The postmortem interval (PMI), corresponding to the period between death and autopsy, ranged from 6 to 28 h in the control group and from 8 to 50 h in the autistic group. The difference in the PMI between the two groups was not significant. The average weight of the brains in the autistic cohort (1,453 g) was not significantly different from that of the control group (1,372 g). The brain hemisphere was fixed with 10% buffered formalin for an average of 408 days in the control and 905 days in

autistic group. Brain dehydration in a series of ascending concentrations of ethyl alcohol for 36 and 38 days in the control and autistic group, respectively, resulted in reduction of brain hemisphere weight by 47% on average in the autistic group and by 45% in control group. Brain hemispheres were embedded in 8% celloidin [Heinsen et al 2000]. Serial 200-µm-thick sections were stained with cresyl violet and mounted with Acrytol.

Morphometry. The morphometric measurements of the material being analyzed were performed without knowledge of the subject's age, gender, clinical diagnosis, or neuropathological status. Neurons were counted using a workstation consisting of an Axiophot II (Carl Zeiss, Goettingen, Germany) light microscope with Plan Apo objectives 1.25x (numerical aperture, N.A., 0.15); 2.5x (N.A. 0.075), and 40x (N.A. 0.75), a specimen stage with a three-axis, computer-controlled stepping motor system (Ludl Electronics; Hawthorne, NY, USA), a CCD color video camera (CX9000 MicroBrightfield Bioscience, Inc., Williston, VT, USA), and stereology software (Stereo Investigator, MicroBrightfield Bioscience, Inc.). The numerical density and total number of neurons per region of interest were estimated using MicroBrightfield software (Fractionator). To eliminate bias related to sectioning defects, 5-µm top and bottom guard zones were applied.

Deleted: o

Anatomical boundaries of examined brain structures and neuron identification for fractionator. Anatomical boundaries of examined brain structures and their cytoarchitectonic subdivisions were described in detail in a previous report [Wegiel et al 2013b]. The neurons were distinguished from glial cells using histological features provided by cresyl violet staining, including neuron size, shape, spatial orientation typical for specific layers, sectors and nuclei; and staining of nuclear heterochromatin, distinct nucleolus in the majority of examined neurons, and staining of the cytoplasm. Small and round nuclei with uniform, intense staining of nuclear chromatin distinguished oligodendrocytes from astrocytes with large, round and pale nucleus with small amount of dispersed chromatin and undetectable nucleolus, and from small microglial cells with a small, usually elongated nucleus [Wegiel et al 2013b].

Sampling. The parameters and procedures that were applied to estimate the numerical density and number of neurons in the 16 brain structures are summarized in Table 2. Brain structures and their subdivisions were delineated at low magnification of 1.25x (47x final mag.), 2.5x (95x), or 5x (190x) to determine the area and the volume of the region of interest using the Cavalieri method. The number of neurons was determined using 40x lens (final magnification, 1,450x). An optical fractionator systemic random sampling scheme was applied (Stereo Investigator, MicroBrightfield). At least four equidistant serial sections were used for neuronal

counts in the anatomical subdivisions of the amygdala (total 12 sections), 10 in the substantia nigra, 14 in cornu Ammonis sectors CA1–CA4, 9 in claustrum, and 16 in the magnocellular basal complex, 6 sections in the thalamus, globus pallidus and nucleus accumbens, 9 in the caudate nucleus and putamen, and 11 in the dentate nucleus. The results of examination of the cytoarchitectonic subdivisions are presented as a mean number of neurons per mm^3 (neuronal density) and total number of neurons per region of interest. The grid size and the virtual counting space were designed for each brain structure to adjust to the size and shape of examined regions. The number of virtual counting spaces ranged from 34 in very small subdivisions, such as the CA4 sector (CE, 0.003), to 1953 in the globus pallidus with a few dispersed neurons (CE, 0.04).

Results

Correlation between the age and volume of brain structures, numerical density and total number of neurons. The volume of examined brain regions, the numerical density and the total number of neurons do not correlate with age in the examined control cohort 4 to 64 years of age and autistic cohort from 4 to 60 years of age. This lack of correlation in both groups might be an effect of a very low representation of subjects over 50 years of age (only 3 subjects in each cohort) but 3.7 times stronger representation of subjects less than 50 years old (11 subjects in each cohort). These data reflect more prominent interindividual differences in brain structure volume than in number of neurons.

The volume of brain subdivisions. The application of the Cavalieri method to estimate volume of 15 brain structures and their 21 subdivisions revealed significant alterations in the nucleus accumbens only (Table 3). The volume of this structure was more in the autistic group (245 mm^3) than in control group (187 mm^3) ($p<0.04$). However, this result might be biased technically because the nucleus accumbens is the only structure in this study with a partially arbitrary delineation of the borderline to estimate volume (the Lehericy et al 1989, method).

The numerical density and total number of neurons. Application of fractionator to determine the numerical density and total number of neurons in 14 brain structures, including the amygdala, thalamus, claustrum, entorhinal cortex, cornu Ammonis, caudate nucleus, putamen, globus pallidus, nucleus accumbens, lateral geniculate body, substantia nigra, magnocellular basal complex, dentate nucleus in the cerebellum and in inferior olive in the brainstem and in their 16 cytoarchitectonic subdivisions (Table 3) revealed significantly lower numerical density of neurons in only three brain regions, including the lateral nucleus in the amygdala (24,282/mm^3

in autism and 27,632/mm^3 in control group; p<0.006), putamen (24,124 mm3 in autism and 27,683 mm3 in control group; p , 0.04) and nucleus accumbens (34,105/mm3 in autism and 40,224/mm3 in control; p<0.01). A significantly lower total number of neurons was found in only one per 30 subregions examined. The mean total number of neurons in the islands of the entorhinal cortex was 21% less in autistic subjects (801,842) than in the control group (1,012,107) (p<0.02). These few deviations from control values may reflect a trend to a broader spectrum of interindividual differences rather than a consistent pattern with clear indication of structures affected.

The range of interindividual differences. All three parameters (structure volume, mm^3; numerical density of neurons, N/mm^3; and total number of neurons/per examined structure) reveal prominent interindividual differences in the control group and these differences are brain-region specific (Table 5). In the control group the mean ratio was 1.93 for structure volume and total number of neurons, and 1.63 for numerical density of neurons. The ratio as a measure of interindividual differences was significantly larger in the autistic group for structure volume (2,42 on average; *P*<0.02). The difference of the ratio for the total number of neurons (2.35) was close to significant range (p<0.06) but for neuronal density (1.79) was not significant. The significant difference (p<0.002) for combined ratios of the volume, numerical density and total number of neurons for the autistic group (2.19) and control subjects (1.83) suggests a broader spectrum of interindividual differences in autistic than in control cohort.

Discussion

The cluster of our studies [Wegiel et al 2010, 2012, 2013b submitted] and this report are different in several aspects than previous postmortem studies of brain pathology in autistic subjects. Thanks to the support of several tissue banks, the Autism Tissue Program/Autism Speaks and families donating tissue, the size of cohorts examined is much larger than in any previous study. The goal of expansion of the size of examined cohorts was to reduce the risk of a small group effect on measures of developmental alterations in a cohort known for heterogeneity and interindividual differences of clinical phenotype and comorbidity. This study was concentrated on subcortical structures, the hippocampus and entorhinal cortex, cerebellum and brainstem, whereas other research groups using part of the same cohort concentrated on detection of developmental alterations in the cerebral cortex [van Kooten et al 2008, Santos et al 2011,

Jacot Descomb et al 2012] and the brainstem superior olive nuclei (auditory system) [Kulesza et al 2011].

In contrast to the traditional focus on localized models of autism pathology, our study of 30 brain regions and their subdivisions is an attempt at detection of a global pattern of developmental abnormalities. Characterization of both the numerical density of neurons and the total number of neurons per region of interest replaces intuitive estimates of neuronal packing density and provides information about total number of neurons per ROI, which can't be estimated without stereological methods.

Evidence for unmodified number of neurons in autism. This study reveals only very limited alterations of the numerical density or total number of neurons in the brain of autistic subjects. Several reports indicated absence of alterations in the number of neurons (Table 6). Coleman et al [1985] study of several cerebral cortical regions did not find alterations in the number of neurons in examined autistic subject. Bailey et al (1998) reported no alterations in neuronal counts in the superior frontal cortex of the six autistic cases. A review of reports characterizing number of neurons shows no alterations in the number of neurons in the BA17, [van Kooten et al 2008] no alterations in the number and density of pyramidal neurons in layers III, V, and VI in the BA44 and 45 [Jacot Descomb et al 2012], and no significant difference in the mean number of pyramidal neurons in the frontoinsular cortex [Santos et al 2011]. The study of von Economo neurons revealed that there is no significant difference in the mean number of von Economo neurons in the frontoinsular cortex in 3 to 41 year-old autistic subjects (Kennedy et al 2007) and unmodified density of von Economo neurons in the anterior cingulate cortex (BA24 a, b, c) [Simms et al 2009]. The study of the frontoinsular cortex in 4 autistic (4 to 11 y) and 3 control subjects (4 to 14 years of age) revealed a 53 % increase in the ratio between von Economo neurons and pyramidal neurons but the difference between the number of von Economo neurons in autistic and control subjects (28,955 and 18,889, respectively) was short of statistical significance ($p<0.054$) and the total estimated number of pyramidal neurons (845 and 807 thousand, respectively) did not reveal a significant difference [Santos et al 2011].

A reduced or unmodified number of Purkinje cells has been reported. Lower Purkinje cell counts have been reported in the cerebellum in four autistic subjects by Ritvo et al (1986). All 9 brains of autistic subjects examined by Kemper and Bauman [1998] showed variable reduction in the number of Purkinje cells. Decreased number of Purkinje cells was also reported in two autistic subjects by Lee et al [2002] but the authors linked these alterations to ischemia rather than developmental defects (Table 7).

Complex studies of the cerebellum revealed that despite genetic and receptor binding evidence of GABAergic system involvement in autism, there is no evidence of abnormalities in the number and distribution of GABAergic basket and stellate interneurons in the cerebellar molecular layer in autistic individuals [Whitney et al 2009]. Whitney et al (2008) study of the number (N/mm of Purkinje cell layer) of calbindin-D28k immunopositive Purkinje cells in crus II in six autistic subjects 13 to 54-years-old and four control subjects 17 to 30-years-old revealed that in three autistic subjects the number was in range of control subjects whereas in three autistic subjects the number of Purkinje cells fell below the control range. Fatemi et al [2002] study of CV stained sections from the cerebellum of 5 autistic males with mean age of 25.2 years (SD 5.26 years) and 5 age-matched controls revealed a 24% decrease in mean Purkinje cell size but no differences in Purkinje cell densities. The study of the cerebellum of a 16-year-old female diagnosed with autism and severe intellectual deficit showed no alterations in the cerebellum [Guerin et al 1996].

Evidence for regionally smaller number of neurons in autism. Estimation with fractionator of the number of neurons in the amygdala in 9 brains of autistic individuals from 10 to 44 years of age and 10 brains of age-matched controls did not reveal a difference in the number of neurons in basal, accessory basal, central and remaining nuclei)[Schumann and Amaral 2006] like in our cohort. However, the authors revealed significantly less neurons in the lateral nucleus (3.47 million in autistic and 4.00 million in control group; $p<0.05$) and in the entire amygdala (10.74 million in autistic and 12.21 million in control group; $p<0.05$). Surprisingly, a significantly ($p<0.006$) reduced number of neurons in the lateral nucleus in the amygdala of autistic subjects in our cohort of 14 subjects 4 to 60 years of age (24,282/mm3 in autism and 27,632/mm3 in control group) suggests developmental alterations in this nucleus. Total number of neurons in the lateral nucleus in autistic subjects was less in autism (4,609,751) than in control group (5,544,098) but the difference did not reach a significant level. Some differences in tissue preservation, as well as selection of epilepsy-free males only, and examination of approximately two times more sections per case (25 in comparison to 12 in our study) may contribute to partially different numbers.

Neuronal loss during the lifespan of autistic subjects.

Some differences between published results of morphometric studies might be related to interindividual differences associated with treatment of epilepsy-related loss of Purkinje cells [Ghatak et al 1976, Rapport and Shaw 1977, Whitney et al 2008], regional loss in response to

chronic traumatic brain injury [Hof et al 1991], neuronal loss associated with enhanced lipofuscin accumulation and oxidative stress [Lopez Hurtado and Prieto 2008] (Table 7), and premortem changes related to the mechanism of death. Detailed neuropathological examination in this project excluded 5 autistic and 4 control cases with premortem and perimortem neuronal loss, as well as with severe autolytic changes [Wegiel et al 2010] to reduce the risk of distortion of results of morphometric studies.

Treatment related loss of Purkinje cells. In some cases Purkinje cell loss can be associated with treatment of epilepsy with phenytoin (Dilantin®). Purkinje cell death is observed throughout the anterior and posterior lobes and in the flocculus/nodulus [Ghatak et al 1976, Rapport and Shaw 1977]. Phenytoin exposure produces ataxia, dysarthria, hypotonia, and intenion tremor [Ghatak et al 1976, McLain et al 1980]. Absence of these symptoms may help to exclude phenytoin as a potential cause of Purkinje cell loss in autistic subjects with a history of phenytoin treatment [Whitney et al 2008].

Evidence for regional neuronal loss in response to chronic traumatic brain injury. In autistic subjects with severe self-injurious behavior, neuronal loss might be caused by neurodegeneration such as described by Hof et al [1991] in a 24-year-old autistic woman (Table 7). Her self-injurious behavior observed since childhood included head-banging. A postmortem study revealed numerous neurofibrillary tangles in the perirhinal and entorhinal cortex, inferior temporal gyrus, and less numerous in the amygdala.

Potential link between neuronal metabolic alterations and loss of neurons in childhood. The study of autistic males 7 to 44 years of age revealed a decline of neuronal numerical density in 7-10 year olds and minimal further decline in adolescence and adulthood in the Wernicke's area (BA22) involved in speech recognition, and gyrus angularis (BA39) involved in reading. Decline associated with enhanced lipofuscin accumulation and increased numerical density of astrocytes was considered associated with oxidative stress (Lopez Hurtado and Prieto, 2008).

Loss of neurons related to mechanisms of death. In some cases medical examiner records reveal a link between drowning, resuscitation and survival for several days on respirator, very severe neuronal loss, gliosis and neovascularization. However, in cases with a less obvious link between ischemia, anoxia, hypoxia and neuronal loss, or in cases with poor records regarding cause and mechanism of death, bank records may be unable to help identify the cause of neuronal death. In studies of small tissue blocks assigned to morphometric studies the disease related pathology might be not discriminated from peculiar pre-mortem alterations.

Evidence for regional higher than normal number of neurons. In contrast to the majority of reports showing no evidence of altered number of neurons, Courchesne et al (2011) revealed 67% more neurons in the prefrontal cortex in autistic 2 to 16 year-old autistic subjects suggesting prenatal disturbances of mechanisms that govern proliferation, cell cycle regulation and apoptosis. Confirmation of these striking differences may indicate that alterations are region-specific and direction of change is opposite in one subregion of the neocortex (increase) than in one subdivision of the amygdala (decrease).

Closing remarks. This study results in a surprisingly strong support for previous reports indicating that the total number of neurons and numerical density of neurons are not modified in the majority of examined brain regions, and developmental alterations are limited to a few regions including reduced number in the lateral nucleus in the amygdala of autistic subjects. Increased spectrum of interindividual differences in the volume of brain subdivisions, numerical density of neuron and total number of neurons are indicators of discrete developmental alterations within the autistic cohort. However, the study of this cohort revealed focal defects of neuronal migration, proliferation and dysplastic changes, again with striking interindividual differences [Wegiel et al 2010, 2012]. The most uniform alteration appears to be (a) significant neuronal soma volume deficit (small neurons) in all 16 examined brain regions in 4 to 8 year old autistic children, and (b) significant correction of neuron soma volume resulting in decrease of differences to only 6 regions in 11 to 23-year-old autistic individuals, and 5 regions in autistic adults (39 to 60 years old). Very limited signs of alterations in the number of neurons but common and significant alterations in the trajectory of neuron size may indicate that brain-region and neuron-type-specific modification of the trajectory of neuron growth are major contributors to desynchronized growth of interacting neuronal circuits and brain structures and to complex clinical phenotype of autism.

Table 1. Tissue samples, demographics of autistic and control subjects, brain weight and changes during processing

Group	Case number	Sex	Age (years)	PMI (h)	Brain weight (g)	H	Fixation (days)	Dehydration (days)	Weight loss (%)
A	425-02	M	4	30	1,280	R	4,560	28	49
A	443-02	F	5	13.2	1,390	R	1,568	28	52
A	M6-06	M	7	25	1,610	R	330	37	55
A	M5-03	M	8	22.2	1,570	R	196	36	45
A	427-02	F	11	12.9	1,460	L	308	34	52
A	445-02	M	13	8	1,470	L	75	33	39
A	M5-05	F	17	25	1,580	L	470	36	51
A	M1-03	F	21	50	1,108	R	136	35	43
A	M7-08	M	22	25	1,375	R	1,034	39	38
A	93-01	M	23	14	1,610	R	533	45	60
A	M7-07	M	36	24	1,480	R	721	37	44
A	M14-10	M	52	27.6	1,625	R	1,650	39	40
A	M14-06	M	56	3.3	1,570	R	692	38	52
A	M13-08	M	60	26.5	1,210	R	398	39	38
Average				**21.9**	**1,453**		**905**	**36**	**47**
SD				**11.4**	**162**		**1,160**	**4**	**7**
C	17-06	F	4	17	1,530	R	126	41	49
C	426-02	F	4	21	1,222	R	233	30	43
C	M15-06	M	7	12	1,240	R	130	41	51
C	M3-04	F	8	20	1,222	R	650	36	51
C	M9-03	M	14	20	1,464	R	1,067	38	44
C	M16-06	F	15	9	1,250	R	372	41	49
C	M1-07	F	20	9	1,340	R	245	37	52
C	444-02	M	23	6	1,520	R	95	45	41
C	M12-04	M	29	13	1,514	R	89	41	49
C	M6-07	M	32	24	1,364	R	460	37	42
C	M10-03	M	48	24	1,412	L	215	38	39
C	252-02	M	51	18	1,450	L	1,819	20	26
C	M14-03	M	52	13	1,430	L	158	47	48
C	M4-07	M	64	28	1,250	R	52	37	51
Average				**16.7**	**1,372**		**408**	**38**	**45**
SD				**6.6**	**118**		**491**	**7**	**7**

PMI, postmortem interval; h, hours; H, hemisphere; R, right; L, left; SD, standard deviation.
Fixation: duration (in days) of fixation in 10 % buffered formalin.
Dehydration: duration (in days) of dehydration in ethyl alcohol.
Weight loss: decrease of hemispheric brain sample weight during dehydration in ethyl alcohol.

Table 2. Parameters and procedures applied to estimate the volume of brain subdivisions and the number of neurons

Structures and their subdivisions	Number of sections examined	Obj. (Cav)	Obj. (Fract)	Grid size (μm) for Fractionator	Test area frame x height (μm)	Number of counting spaces	Number of neurons counted	CE*
Amygdala	12	1.25x	40x	1000x1000	80x80x10	356	454	0.01
Thalamus	6	1.25x	40x	1000x1000	80x80x10	188	313	0.01
Entorhinal c. LII	6	2.5x	40x	400x400	60x60x30	109	117	0.003
LIII				800x800	60x60x30	117	139	
LV				600x600	60x60x30	62	122	
LVI				800x800	60x60x30	67	124	
CA1	14	2.5x	40x	1000x1000	60x60x30	53	139	0.003
CA2				400x400	60x60x30	66	122	
CA3				400x400	60x60x30	79	118	
CA4				600x600	100x100x30	34	130	
Caudate nucleus	9	2.5x	40x	1400x1400	60x60x30	270	C725 A776	0.04
Putamen	9	2.5x	40x	1400x1400	60x60x30	309	C848 A 841	
Globus pallidus	6	2.5x	40x	300x300	80x80x30	1953	C539 A471	
Nucleus accumbens	6	2.5x	40x	700x700	60x60x30	202	C717 A 781	
Magnocellular LGB	4	5x	40x	500x500	100x100x30	96	230	0.002
Parvocellular LGB	4	5x	40x	500x500	100x100x30	134	1,418	0.002
Claustrum	13	1.25x	40x	250x250	60x60x10	274	318	0.01
Substantia nigra	10	2.5x	40x	500x500	80x80x30	366	C485 A 502	0.002
Magnocellular basal complex (Ch1-Ch4)	16	2.5x	40x	500x500	80x80x30	323	C530 A 483	0.005
Dentate nucleus	11	2.5x	40x	1000x1000	180x180x30		792	0.002
Inferior olive	4	2.5x	40x	1000x1000	180x180x30	132	260	0.002

*CE, the average predicted coefficient of error of the neuronal counts (Scheaffer).
Obj. (Cav), Objective lens (1,25x, 2.5x or 5x) used to provide final magnification for planimetry (Cavalieri).
Obj. (Fract), Objective lens (40x) used for fractionator to estimate neuronal density and total number
Number of sections examined – mean number of equal distance sections examined per structure/case.

Table 3. The volume of 15 brain structures and 21 cytoarchitectonic subdivisions in one brain hemisphere of autistic and control subjects

Structure/cytoarchitectonic subdivision	Volume (mm^3)		
	Autism	Control	*P* <
Amygdala	397	405	ns
- Basal n.	113	116	ns
- Lateral n.	189	202	ns
- Accesory basal n.	82	76	ns
- Central n.	13	11	ns
Thalamus	3,030	2,999	ns
Claustrum	432	442	ns
- Insular claustrum	238	241	ns
- Prepiriform claustrum	194	201	ns
Entorhinal cortex (EC)	591	616	ns
- EC Islands	38	42	ns
- EC III	247	251	ns
- EC V	58	65	ns
- EC VI	133	133	ns
Cornu Ammonis (CA)	598	601	ns
- CA1	439	426	ns
- CA2	32	37	ns
- CA3	44	47	ns
- CA4	84	90	ns
Caudate nucleus	2,157	1,880	ns
Putamen	2,614	2,379	ns
Globus pallidus	613	586	ns
- External globus pallidus	412	384	ns
- Internal globus pallidus	201	202	ns
Nucleus accumbens	245	187	0.04
Lateral geniculate body	80	77	ns
Substantia nigra	101	95	ns
Magnocellular basal complex	81	85	ns
Dentate nucleus	245	232	ns
Inferior olive	62	59	ns
Cerebellum	31,507	31,133	ns
- Cerebellar cortex	24,603	24,811	ns
- Molecular layer	12,487	12,885	ns
- Granule cell layer	12,116	11,926	ns
- Cerebellar white matter	6,556	6,005	ns
- Cerebellar nuclei	348	317	ns

Table 4. The numerical density (N/mm^3) and total number of neurons per structure in 14 structures and 16 cytoarchitectonic subdivisions in one hemisphere of autistic and control subjects

Structure/cytoarchitectonic subdivision	Autistic, control subjects	Numerical density			Total number of neurons per region		
		Autism	Control	$p <$	Autism	Control	$p <$
Amygdala	14, 14	24,974	27,312	ns	9,845,142	11,003,603	ns
- Basal nucleus	14, 14	25,203	26,436	ns	2,821,429	3,154,350	ns
- Lateral nucleus	14, 14	24,282	27,632	0.006	4,609,751	5,544,098	ns
- Accesory basal nucleus	14, 14	25,216	28,080	ns	2,046,439	2,109,385	ns
- Central nucleus	14, 14	26,984	29,922	ns	342,122	330,766	ns
Thalamus	14, 14	15,677	16,458	ns	47,403,083	49,291,830	ns
Claustrum	13, 14	28,923	29,590	ns	12,386,770	12,994,709	ns
- Insular claustrum	13, 14	27,895	28,219	ns	6,576,940	6,729,652	ns
- Prepiriform claustrum	13, 14	29,974	30,247	ns	5,774,148	6,187,932	ns
Entorhinal cortex (EC)	13, 14	25,726	27,777	ns	11,640,072	13,114,065	ns
- EC Islands	13, 14	21,725	24,271	ns	801,842	1,012,107	0.02
- EC III	13, 14	22,440	24,772	ns	5,522,613	6,139,719	ns
- EC V	13, 14	31,864	32,483	ns	1,831,442	2,061,207	ns
- EC VI	13, 14	26,877	29,583	ns	3,484,175	3,901,033	ns
Cornu Ammonis (CA)	14, 12	25,929	24,789	ns	14,815,244	15,047,032	ns
- CA1	14, 12	27,182	27,461	ns	11,575,341	11,460,373	ns
- CA2	14, 12	34,202	32,254	ns	1,051,901	1,162,676	ns
- CA3	14, 12	24,899	25,094	ns	1,057,981	1,170,497	ns
- CA4	14, 12	13,835	14,348	ns	1,130,021	1,253,487	ns
Caudate nucleus	14, 14	25,378	26,912	ns	54,693,459	49,292,390	ns
Putamen	14, 14	24,124	27,683	0.04	61,675,039	65,111,021	ns
Globus pallidus	14, 14	2,097	2,340	ns	1,277,936	1,378,086	ns
- External globus pallidus	14, 14	2,281	2,547	ns	899,307	904,365	ns
- Internal globus pallidus	14, 14	1,913	2,081	ns	378,629	407,897	ns
Nucleus accumbens	14, 14	34,105	40,224	0.01	8,078,093	7,477,977	ns
Lateral geniculate body	14, 13	18,730	18,129	ns	1,465,939	1,379,552	ns
Substantia nigra	12, 14	7,113	7,676	ns	710,829	707,228	ns
Magnocell basal complex	14, 14	8,982	9,445	ns	768,415	790,893	ns
Dentate nucleus	14, 14	4,065	4,018	ns	970,440	919,572	ns
Inferior olive	10, 11	12,811	12,289	ns	793,002	715,496	ns

Table 5. Interindividual differences. The ratio between the lowest and highest volume (mm^3), numerical density of neurons (n/mm^3) and total number of neurons (N/structure) in autistic and control group. Bold, higher ratio.

Brain structure	Structure volume		Numerical density of neurons		Total number of neurons	
	Autism	Control	Autism	Control	Autism	Control
Amygdala	532:168 =**3.17**	617:293 =2.10	32,814:19,804 =**1.66**	33,460:24,121 =1.39	15,333,103/3,455,988 =**4.44**	16,305,101/8,710,689 =1.87
Thalamus	3,965:2,145 =**1.85**	3,925:2,510 =1.56	21,179:12,861 =**1.64**	18,488:14,520 =1.27	70,801,397:40,602,177 =**1.74**	61,389,874/40,060,680 =1.53
Claustrum	773:285 =**2.71**	631:288 =2.19	37,477:22457 =1.67	37,466:21,343 =1.75	20,003,694:7,643,700 =**2.62**	18,988,052:8,674,878 =2.18
Entorhinal Cortex	776:400 =**1.94**	805:445 =1.81	29,660:21,487 =1.38	33,843:24,914 =1.36	15,823,200:8,750,500 =1.81	16,748,853:9,345,060 =1.79
Cornu Ammonis	915:399 =**2.29**	880:486 =1.81	36,375:18,149 = **2.00**	30,450:20,039 =1.52	18,929,878:10,611,215 =**1.78**	18,503,749:12,696,457 =1.46
Caudate Nucleus	2,697:1,491 =1.80	2,765:1,085 =**2.54**	32,650:17,161 =**1.90**	34,917:21,329 =1.64	83,518,159:33,056,686 =2.53	73,421,810:26,991,452 =**2.72**
Putamen	3,858:1,820 =**2.11**	3,160:1,858 =1.70	29,470:14,265 =**2.06**	37,755:21,669 =1.74	77,625,968:36,685,004 =**2.12**	82,791,656:51,822,048 =1.60
Globus Pallidus	874:400 =**2.18**	754:436 =1.73	2,585:1,105 =**2.34**	3,127:1,633 =1.91	1,704,953:823,165 =**2.07**	1,708,394:1,105,284 =1.55
Nucleus accumbens	501:130 =**3.85**	258:124 =2.08	45,226:27,041 =**1.67**	54,247:33,805 =1.60	13,547,336:5,886,729 =2.3	11,858,832:4,903,753 =2.**42**
Lateral geniculate body	115:53 =**2.17**	97:57 =1.70	23,429:15,281 =1.53	20,968:12,420 =**1.69**	1,833,960:1,151,880 =1.59	1,690,410:975,270 =**1.73**
Substantia Nigra	156:64 =**2.44**	117:69 =1.69	8,913:5,561 =1.60	12,368:5,935 =**2.08**	1,148,757:540,855 =**2.12**	913,165:581,538 =1.57
Magnocell basal complex	143:44 =**3.25**	122:51 =2.39	11,122: 6,487 =**1.71**	10,882:6,919 =1.57	1,193,342:417,296 =**2.85**	1,154,418:543,730 =1.95
Dentate Nucleus	364:130 =**2.80**	289:161 =1.79	6,859:2,847 =**2.41**	5,295:2,827 =1.87	1,482,716:472,840 =**3.13**	1,243,210:511,111 =2.43
Inferior Olive	66:50 =1.32	74:37 =**2.00**	15,509:9,846 =**1.57**	15,194:10,546 =1.44	1,043,786:573,631 =1.82	956,813:456,098 =**2.10**
Mean ratio	**2.42**	1.93	**1.79**	1.63	**2.35**	1.93

Table 6. Review of reports characterizing developmental alterations of the number of neurons in autistic subjects

Region	Topography and type of alterations	Number of subjects and age	Reference
Cortical regions	No alterations of the density of neurons	Autism: 1	Coleman et al 1985
Multi-regional study	Increased neuronal packing density in the CA1-4, subiculum, entorhinal cortex, mammilary body, medial septal nucleus, amygdala (central, medial and cortical nuclei). Variable regional reduction in the density of Purkinje cells in all 9 brains of autistic subjects	Autism 1: (29 y) [1985] Autism 6: (9, 10, 12, 22, 28, 29 y) [1994]. Autism 9: (6, 8, 10, 19, 20, 21, 28, 35, 54 y) [1998] (and age-matched control)	Bauman and Kemper 1985, 1994 Kemper and Bauman 1998
Multi-regional study	Unmodified neuronal density in the superior frontal gyrus in 5 cases and reduced in one case diagnosed with epilepsy. Increased density in the CA in one of 6 autistic subjects. Reduced density of Purkinje cells in 5 cases associated with glia proliferation in 4 cases.	Autism: 6 (4-24 y) Control: 5 (age-matched)	Bailey et al 1998
Amygdala	Less mean number of neurons in the amygdala of autistic (10.4 million) than in control (12.21 million) subjects ($p<0.05$). Less neurons in the lateral n of autistic (3.47 million) than in control (4.00 million) subjects ($p<0.05$).	Autism: 9 (10-14 y) Control: 10 (11-44 y)	Schumann and Amaral 2006
BA 4, 17, 9	Increased neuronal density by 23%.	Autism: 8 (4-24 y) Control: 6 (4-25 y)	Casanova et al 2006
Frontoinsular cortex	No significant difference in the mean number of von Economo neurons in autistic (35,329) and control (29,125) subjects.	Autism: 4 (3-41 y) Control: 5 (2-75 y)	Kennedy et al 2007
Fusiform gyrus (FG); Occipital cortex (BA17)	FG: Reduced mean number of neurons in layers III, V, VI by 23.7%, 14.3%, 10.6%, respectively. BA17: No alterations in the number of neurons.	Autism: 7 (4-23 y) Control: 10 (3-74y)	Van Kooten et al 2008 +
Anterior cingulate cortex (BA24a, b, c)	Significant decrease of the neuronal packing density in layers V and VI of BA24c. No difference in the density of von Economo neurons	Autism 9 (15-54 y) Control 4 (20-55 y)	Simms et al 2009
Frontoinsular cortex	No significant difference in the mean number of pyramidal neurons (845 thousand in autistic and 807 thousand in control subjects). 53% more von Economo neurons in autistic than in control subjects.	Autism: 4 (4-11 y) Control: 3 (4-14 y)	Santos et al 2011
Dorsolateral and mesial prefrontal cortex (DL-PFC and M-PFC)	67% more neurons in the prefrontal cortex in autistic subjects (1.94 billion) than in control (1.16 billion; $p< 0.002$) subjects. 79% more neurons in DL-PFC and 29% more in M-PFC in autistic than control subjects.	Autism: 7 Control: 6 2-16 y.	Courchesne et al 2011
Frontal inferior (BA44 and 45)	No alterations of the number and density of pyramidal neurons in layers III, V, and VI the BA44 and 45	Autism: 7 (4-52 y) Control: 7 (4-48 y)	Jacot Descomb et al 2012
Purkinje cells	Lower Purkinje cell density	Autism: 4	Ritvo et al 1986
Purkinje cells	No alterations in cerebellum	Autism: 1; 16 y	Guerin et al 1996
Purkinje cells	No difference in mean Purkinje cell densities in the cerebellum of autistic and control subjects	Autism: 5 (25.2y) Control: 5 (24.2 y)	Fatemi et al 2002
Purkinje cells	No difference in the density of calbindin-D28k-positive Purkinje cells, but in three of six autistic subjects the mean density of Purkinje cells (number per mm of Purkinje cell layer) fell below control range.	Autism: 6; 13-54y Control: 4; 17-30y	Whitney et al 2008b
GABAergic interneurons in the cerebellar c.	No abnormality in the mean density of GABAergic basket and stellate interneurons (parvalbumin-positive) in the molecular layer in the cerebellar cortex	Autism: 6; 13-54y Control: 4; 17-30y	Whitney et al 2009
Brainstem - superior olive nuclei	Significantly less mean number of neurons in the lateral superior olive (by 67%), medial nucleus of the trapezoid body (by 45%), superior paraolivary nucleus (by 57%) in autistic subjects compared to controls.	Autism: 9; 2-36 y Control: 4; 8-32 y	Kulesza et al 2011

Table 7. Neuronal loss in autistic subjects related to known or hypothetical mechanisms

Region	Neuronal loss	Number of subjects and age	Reference
Multiregional neurodegeneration	Neurofibrillary degeneration and neuronal loss in the cortex, amygdala, prepiriform and orbito-frontal cortex of autistic female with self-injuries behavior including head banging – neuronal loss related to severe and chronic brain injury.	Autism: 1 (24 y)	Hof et al 1991
Purkinje cells	Purkinje cell loss with granule cell loss and gliosis, possibly of ischemic origin, in two of eight autistic subjects.	Autism: 8 (24.6 y) Control: 10 (27.9 y) (mean age)	Lee et al 2002
Language-related cortex	Decline of neuronal density in BA22 (Wernicke's area), BA39 (angular gyrus) and BA44 (part of the Broca's area) observed in parallel with enhanced lipofuscin accumulation and oxidative stress.	Autism: 8 (7-44 y) Control: 7 (8-56 y)	Lopez-Hurtado and Prieto 2008

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