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

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14. ABSTRACT In this program project, 56 brains were examined, including 22 brains of subjects with idiopathic autism (unknown etiology), 12 brains of individuals with autism associated with chromosome15 duplication (dup15) and 22 brains of control subjects from 2 to 65 years of age. The study of a global pattern of brain developmental abnormalities integrates results of morphometric study of 17 brain structures in autistic and control subjects. Significant similarities of developmental alterations of neuronal proliferation, migration, and cytoarchitecture in idiopathic autism and autism caused by dup(15) indicate that, in part, developmental defects are caused by similar mechanisms regardless of the etiological factor. However, (a) the absence of cortical dysplasia, (b) presence of a several fold more frequent pathology in the dentate gyrus, (c) more intraneuronal Aβ in neurons, (d) a very high prevalence of early onset of seizures in individuals with autism dup(15) compared to the idiopathic autism subjects indicate that the etiology has a specific contribution to structural, biochemical and functional changes in autism. Striking brain region specific delays of neuronal growth in children 3-8 years of age, indicate that autism is caused by failure of mechanisms controling neuron and brain growth. Regional dysregulation results in desynchronization of growth of interacting neurons, neuronal circuits, and neurotransmitter systems. 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. Enhanced APP processing with α- and γ-secretases, leading to enhanced accumulation ofAβ in neuronal cytoplasm observed in the majority of autistic subjects, including children, is an early sign of an altered non-amyloidogenic pathway of APP processing. Early onset of diffuse plaques (at age of 39, 51 and 52 yrs) indicates increasing risk of early activation of amyloidogenic pathway of APP processing in autism with all consequences of intracellular and extracellular accumulation of toxic, oligomerized and fibrillized Aβ. This study shows that autism has age-specific manifestations of altered mechanisms leading to structural and functional changes.

15. SUBJECT TERMS Autism, Developmental Delay of Neuronal Growth, Desynchronization of Brain Development

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Table of Contents
Subproject 2 JW
Annual Report

Page

Annual Report #3
August 19, 2011

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.

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 is to establish correlations between morphological and biochemical markers of autism and clinical symptoms of disease. To achieve these goals, we proposed three subprojects. The factor integrating these three closely collaborating groups is the concentration of a broad spectrum of aims and methods on brains of 56 subjects including: 22 brains of autistic people, 12 brains of individuals with autism associated with chromosome15 duplication (dup15) and 22 brains of control subjects.

This Program Project is focused on the detection of:
(a) mechanisms leading to morphological changes and the clinical autism phenotype,
(b) morphological and biochemical markers of autism,
(c) correlations between pathology and clinical manifestations of autism, and
(d) those pathological domains that might be a target for treatment.

Progress of work is consistent with the original Program Project and Project 1 aims and timetable.

Material : We examined 56 brains including 22 brains of autistic subjects, 12 brains of individuals with autism associated with chromosome15 duplication (idic15) and 22 control subjects from 2 to 65 years of age. The neuropathological criteria were established in cooperation with Project 1. Cases not meeting the ADI-R criteria and cases with signs of comorbidity, perimortem 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. Consistent with the Statement of Work, we examined four brain structures and their

subdivisions (for a total of 9 brain subregions), most likely affected by developmental delay and metabolic aberration in adults and aged people with autism:
(1) Amygdala: lateral, latero-basal, accessory basal and central nuclei (aggression, fear, anxiety, memory, cognition).
(2) Caudate nucleus, (3) putamen, (4) globus pallidus, (stereotypes, rituals).
(5) N. accumbens ("social brain", reward system).
(6) Nucleus supraopticus and **(7) N. paraventricullaris**. These 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 (language, pointing, motor functions, cognition).
(9) Dentate nucleus (language, pointing, motor functions, cognition).

However, we found that to achieve a more global view on brain developmental alterations we needed to expand the list of examined brain regions by adding eight brain structures:
(1) Entorhinal cortex (input to the memory system)
(2) Hippocampal formation (memory system; processing and storage of cortical data)
(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) 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).

After these expansions, the study of a global model of brain developmental abnormalities integrates 17 localized models of developmental alterations in the brain of autistic and control subjects.

Metabolic alterations in autism. Enhanced beta-amyloid precursor protein (APP) processing with α-secretase, was reported in an autistic cohort (Sokol et al, 2007). Bailey et al, (2008) reported a significant increase of secreted APP (sAPP-α) levels in the blood plasma in 60% of the autistic children. These studies support the hypothesis that increased APP processing in the α-secretase pathway takes place in autism, and that the plasma levels of sAPP-α may be an early biomarker, of at least a subgroup, of children with autism (Bailey et al, 2008).

We found that an 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 and that enhanced accumulation is brain region and cell type specific. These developmental alterations are more severe in the brains of idic15 subjects diagnosed with autism and epilepsy than in subjects with idiopathic autism. Abnormalities of Aβ intracellular accumulation in an early stage of brain development suggest their link to the clinical phenotype, including seizures, in idiopathic autism and autism associated with idic15. Detection of amyloid plaques in older autistic subjects suggests that abnormal brain development is associated with abnormal maturation and aging.

Two papers are ready for submission and two others are in preparation for submission. To complete the study of 17 regions and expand studies of metabolic changes, we are asking for a no cost extension of Project 2 and the other two Projects (1-Dr Thomas Wisniewski and 3 – Dr Abha Chauhan).

Outcome: Historically, this is the largest postmortem morphological, morphometric, and biochemical multidisciplinary study integrating efforts of several groups concentrated on the link between etiology, genetic defects, developmental and age-associated changes of brain structure and metabolism contributing to clinical phenotype of autism (See: Key Research Accomplishments and Conclusions).

BODY

SUBPROJECT 1: DEFECTS OF CHOLINERGIC NEURONS DEVELOPMENT IN AUTISM

BACKGROUND

Pattern of developmental abnormalities in the brain of autistic subjects. Autism is associated with signs of curtailed neuronal development in many brain regions (Bauman and Kemper, 1985), developmental alterations of the neocortex including abnormal structure of minicolumns (Casanova et al., 2002), and defects of neuronal proliferation, migration, and dysplastic changes (Wegiel et al., 2010). Our study of the hippocampus, entorhinal cortex, amygdala, caudate, putamen, nucleus accumbens, substantia nigra, thalamus, claustrum, cerebellum and inferior olive revealed (a) brain region and neuron type specific rate of delay of neuronal growth and (b) partial or complete correction of neuron size in late childhood or adulthood in majority of affected structures. However, the study of the substantia nigra and the cornu Ammonis, revealed unmodified size of neurons in autistic subjects. It suggests, that the clinical phenotype of autistic subjects is a reflection of desynchronized development of brain circuits and neurotransmitter systems.

Nucleus basalis of Meynert (NBM) function. NBM consists of four major nuclei that send cholinergic, GABAergic and glutamatergic axons to the cortical mantle, amygdala, and many subcortical structures contributing to the clinical phenotype of autism. Cholinergic drive to the forebrain plays a modulatory role in anxiety, arousal and attention, and is essential for many learning and memory tasks (Murray and Fibiger, 1985; Kilgard, 2003).
Ch4 complex act as the cholinergic relay for transmitting limbic and paralimbic information to the neocortex 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 convergance.

AIM

The aim of this study is to determine whether the cholinergic system of autistic children is affected by developmental delay and contributes to the clinical phenotype of autism.

MATERIAL AND METHODS

Material. Twelve brain hemispheres of autistic subjects from 4 to 60 years of age and 12 control subjects from 4 to 64 years of age were preserved for neuropathological and morphometric studies. Four age-matched pairs of autistic and control subjects represented the youngest group (4/4, 5/4, 7/7, and 8/8 years of age). Eight pairs represented late childhood and adulthood (13/14, 17/15, 21/20, 23/23, 22/29, 36/32,56/52, 60/64). In the autistic and control groups, the proportion between males and females was 9:3 and 7:5, respectively.

Histology. Brain hemispheres were fixed in 10% formalin, dehydrated in ascending concentrations of ethyl alcohol, and embedded in celloidin. Free floating, 200 μm-thick serial sections were stained with cresyl violet (CV) and used for neuropathological evaluation and morphometric studies.

Neuropathology. Neuropathological study of approximately 120 coronal hemispheric CV-stained sections per case revealed autism-associated developmental abnormalities, including defects of migration (heterotopias), focal defects of cytoarchitecture (dysplasia), or abnormal subependymal proliferation in 92% of subjects. During selection of 12 autistic and 12 control subjects five brains of autistic and four brains of control subjects were excluded due to premortem pathology or postmortem changes.

Clinical inclusion criteria. Inclusion of the brain to morphological studies was based on Autism Diagnostic Interview – revised (ADI-R).

Morphometric methods and statistical analysis

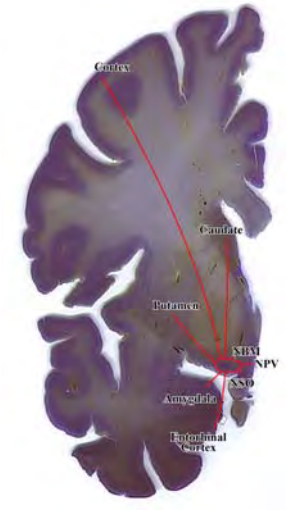
The fractionator method was used to determine the number of neurons, the Cavalieri method to estimate the volume of the NBM subdivisions, and Nucleator method to determine the volume of neurons and neuronal nuclei within four NBM subdivisions in 12 autistic and 12 control subjects.

For morphometry, the image analyzer with computer-controlled automatic stage installed on Axiophot II microscope (Zeiss) and Stereo-Investigator and nucleator software (Microbrightfield, VT, USA) were used. The Nucleator was applied at final magnification 1,480 x (objective lens: 40 x). In the autism and control groups, the mean number of examined neurons was 151 and 156, respectively, and the Schaffer coefficient of error was less than 0.01.

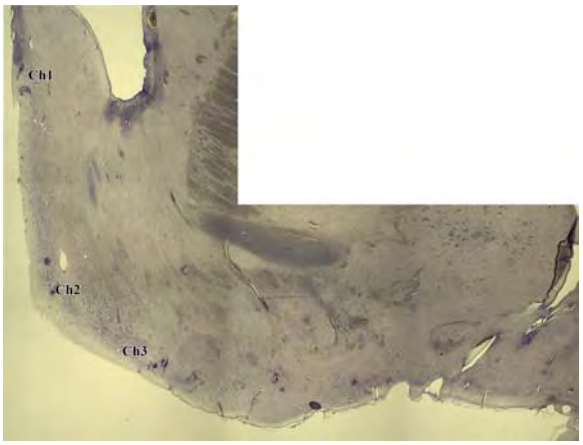
The NBM subdivisions volume, number of neurons, and mean volume of neurons and neuronal nuclei were compared in autistic and control cohorts in repeated-measures ANOVA.

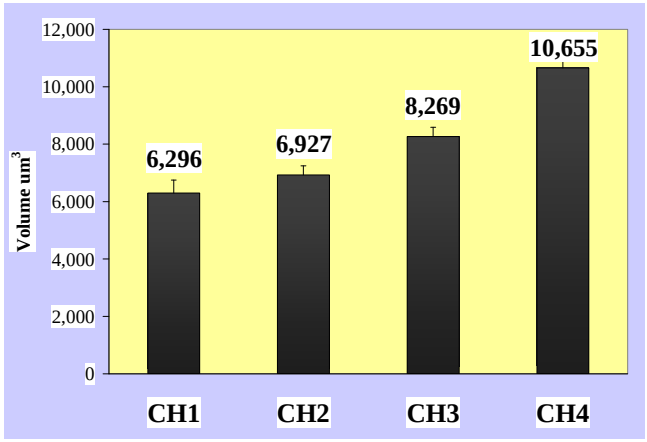
Nucleus basalis of Meynert connectivity

Large NBM neurons project to the entire cortex, including archicortex (entorhinal cortex), as well as to the hippocampus, amygdala, thalamus, hypothalamus (nucleus supraopticus – NSO; nucleus paraventricularis – NPV), nucleus accumbens, caudate nucleus, putamen, globus pallidus and brainstem. The amygdala projects heavily to Ch4 and receives substantial input from Ch4 neurons.



Nucleus basalis of Meynert cytoarchitecture
Based on topography, expression of neurotransmitters, connectivity, and size of neurons four major NBM anatomical subregions (Ch1-4) could be distinguished:
Ch1 region is composed primarily of small size (6,296 μm^3) round neurons.
Ch2 region is build of moderate size (6,927 μm^3) oval neurons.
Ch3 consists of fusiform irregularly scattered large (8,269 μm^3) neurons.
Ch4 is characterized by a very large (10,655 μm^3) hyperchromatic ellipsoid or polygonal neurons.
Most of large neurons in the NBM are cholinergic neurons. Most of small neurons are non-cholinergic neurons positive for enkephalins, neurotensin,
oxytocin, somatostatin, and vasoactive intestinal polypeptide.





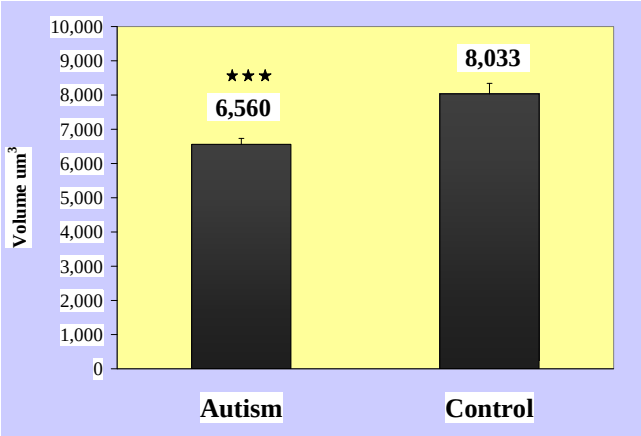
Number of neurons in the NBM subdivisions. Ch4 region, with 65% of all NBM neurons, is the largest component of human NBM. Regions Ch1, 2, and 3, contribute to NBM neuronal population in only 6%, 10%, and 19%, respectively. Similar proportions were found in both, control and autistic subjects.

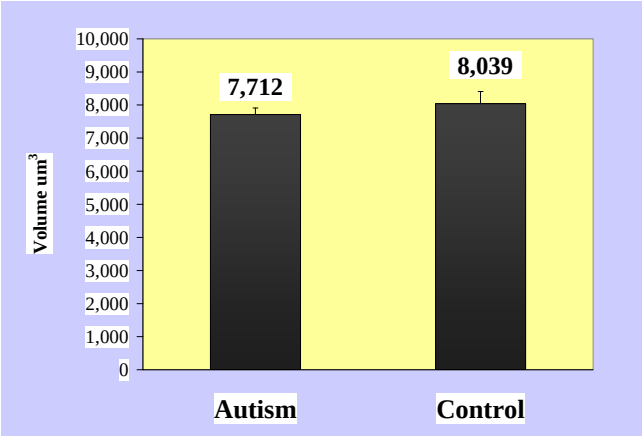
Total volume of the NBM. The mean total volume of all four NBM subdivisions was comparable in autistic (79 mm^3) and control subjects (85 mm^3) from 4 to 64 years of age.

Neuronal density in the NBM
The mean neuronal density was insignificantly less in autistic (9,053/mm^3) than in control subjects (9,285/mm^3) from 4 to 64 years of age.

Total number of neurons in the NBM. The total number of neurons was also insignificantly less in autistic (703,924) than in control (778,316) subjects from 4 - 64 years of age.

Reduced neuron volume in 4-8-year-old autistic subjects. General linear models, with age group (4-8 years vs. over 8-years of age) as a between-subject effect, and autistic status and the interaction of autistic status and age groups as within-subject effect, were used to examine the combined effects of age and autistic status on neuronal and nuclear volume.
Autistic subjects had reduced neuronal volume ($F = 13.161$, $p = .005$); this was also a non-significant trend for younger subjects ($F = 3.942$, $p = .075$). A significant interaction of younger age and autistic status was observed ($F = 5.395$, $p = .043$), indicating that the association between autism and a reduced volume of neurons was most pronounced in the youngest subjects.
Mean neuron volume in 4 to 8-year-old autistic subjects (6,560 μm^3) was 19% less than in control subjects (8,033μm^3, $p < 0.007$).





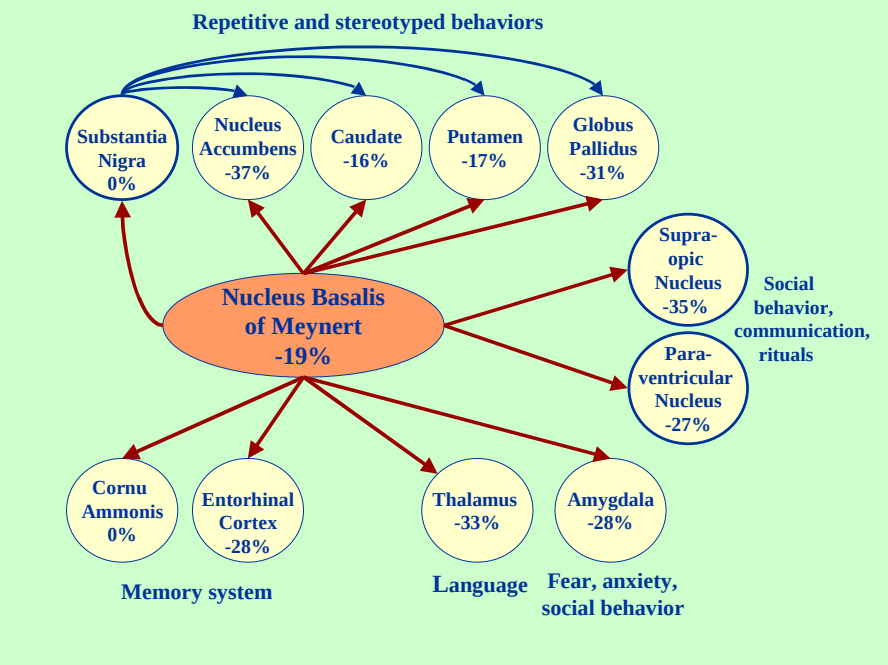
The difference between mean neuron volume in autistic (7.712 μm^3) and control subjects (8,039 μm^3) more than 8 years of age was not significant.

Reduced mean neuronal nucleus volume in 3-8 y old autistic subjects. Autistic subjects also had reduced nuclear volume ($F = 15.434$, $p = .003$). A significant interaction of younger age and autistic status was observed for nuclear volume as well ($F = 8.169$, $p = .017$), indicating that the association between autism and reduced nuclear volume was also most pronounced in the youngest subjects.

Mean neuronal nucleus volume in 4 to 8-year-old autistic subjects (462 μm^3) was 25% less than in control subjects (619 μm^3, $p < 0.05$).

The difference between mean neuronal nucleus volume in autistic (518 μm^3) and control subjects (554 μm^3) more than 8 years of age was not significant.

SUMMARY AND CONCLUSIONS



This study of the nucleus basalis of Meynert is a component of research of a global pattern of developmental delay of neuronal growth and correction of neuron size in late childhood/adulthood in the brain of autistic subjects.

1. Detected delay of NBM neurons growth by 19% and their nuclei by 25% at age of 4 to 8 years, may reflect defective function of cholinergic system in early childhood.
2. Reduced volume of neurons in the NBM of autistic subjects may result in altered cholinergic innervation of the cortical mantle and contributing to anxiety, arousal, deficit of attention, and learning difficulties.
3. Abnormal cholinergic innervation may affect 10 examined regions with eight revealing similar to NBM significant, brain structure specific delay of neuronal growth.
4. The outcome of combination of: region/cell type specific delay of neuronal growth and systemic defects of cholinergic innervation might be a broad spectrum of autistic phenotypes, including communication and social deficits, and repetitive and stereotyped behaviors.
5. Mechanisms leading to developmental delay of neuronal growth appear to be the target for preventive/therapeutic interventions.

SUBPROJECT 2: THE OLIVO-FLOCCULAR CIRCUITRY DEVELOPMENTAL DEFECTS IN AUTISM

BACKGROUND

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). 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.

AIMS

The aim of this study was to detect and characterize defects of the olivo-floccular circuit that may contribute to altered oculomotor function in autism.

MATERIAL AND METHODS

Cerebellum and brainstem of 12 autistic and 10 control subjects were examined. One brain hemisphere from each subject was fixed in 10% buffered formalin for a period of time from 6 weeks to several months and dissected into 30 mm thick frontal slabs. After rinsing the tissue blocks were dehydrated in a graded series of ethyl alcohol and embedded in celloidin or in polyethylene glycol (PEG). 200 μm-thick celloidin and 50-μm thick PEG sections were used for morphometry. Free-floating 50 μm-thick serial PEG sections were used for immunostaining.

An expanded neuropathological protocol, based on examination of one cresyl violet (CV) stained section per 0.6 mm in celloidin protocol and per 1 mm in PEG protocol, was used to detect the type, topography and severity of qualitative changes. Application of ADI-R eliminated cases of atypical autism. Application of neuropathological criteria eliminated cases with unrelated pathology, pre- and postmortem changes.

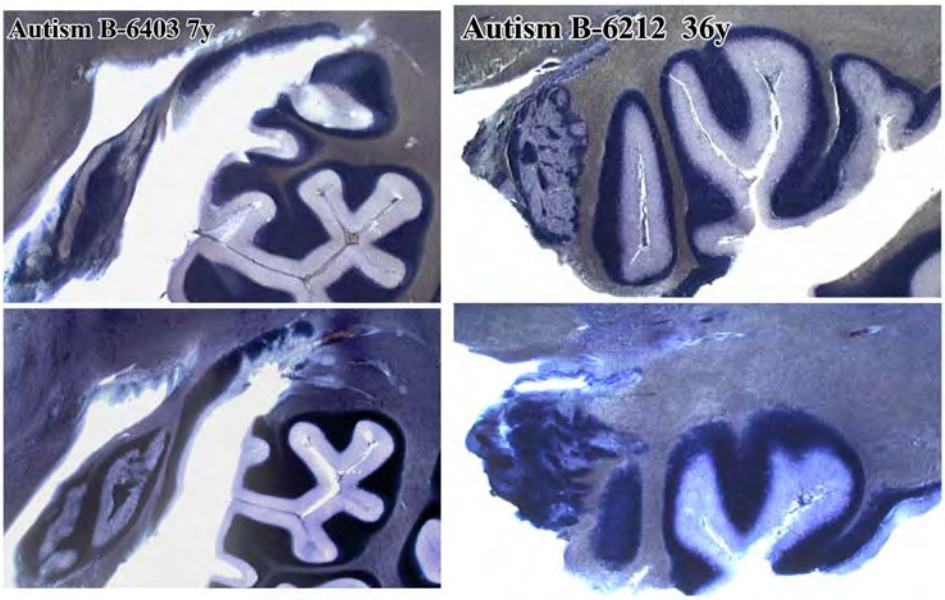
To detect quantitative changes, the image analyzer with computer-controlled automatic stage installed on Axiophot microscope (Zeiss) and the stereology software package (Microbrightfield, VT, USA) were used. The volume of the flocculus was estimated at 48x magnification (objective lens x1.25) by using Stereo-Investigator. The volume of Purkinje cells soma and their nuclei were estimated in the flocculus and in the entire cerebellar cortex at 1,480x magnification (objective lens x40) by using Nucleator.

Calcium binding protein in the soma, dendrites and axons on Purkinje cells and in neurons in the inferior olive was detected with anti-calbindin mouse mAb D-28.

FLOCCULAR DYSPLASIA

Prevalence. Dysplastic changes were found in the flocculus of 8 of 12 autistic subjects (67%). In the control group, a small and medium size dysplasia was found in the flocculus of two of the 10 control subjects (20%).

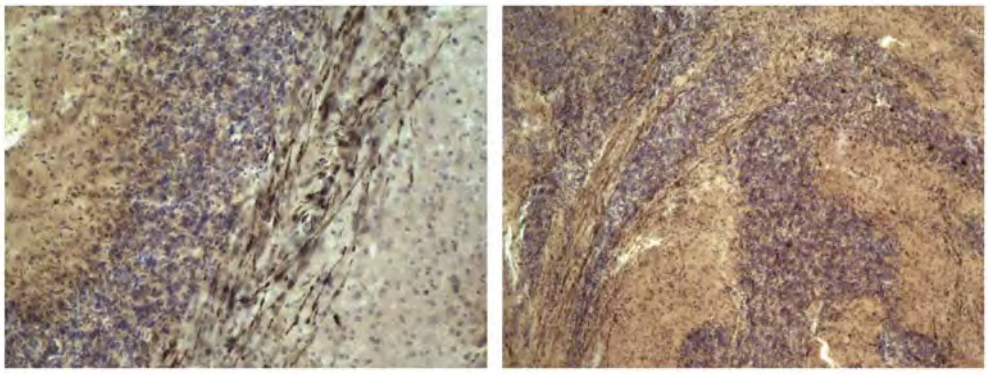
Topography and morphology. Dysplastic changes only affect the rostral portion of the flocculus. In the dysplastic portion of the flocculus laminar organization of the granule, molecular and the Purkinje cell layer, as well as, white matter is profoundly distorted. Serial sections illustrate topography and morphology of dysplastic changes in the flocculus of three autistic subjects.



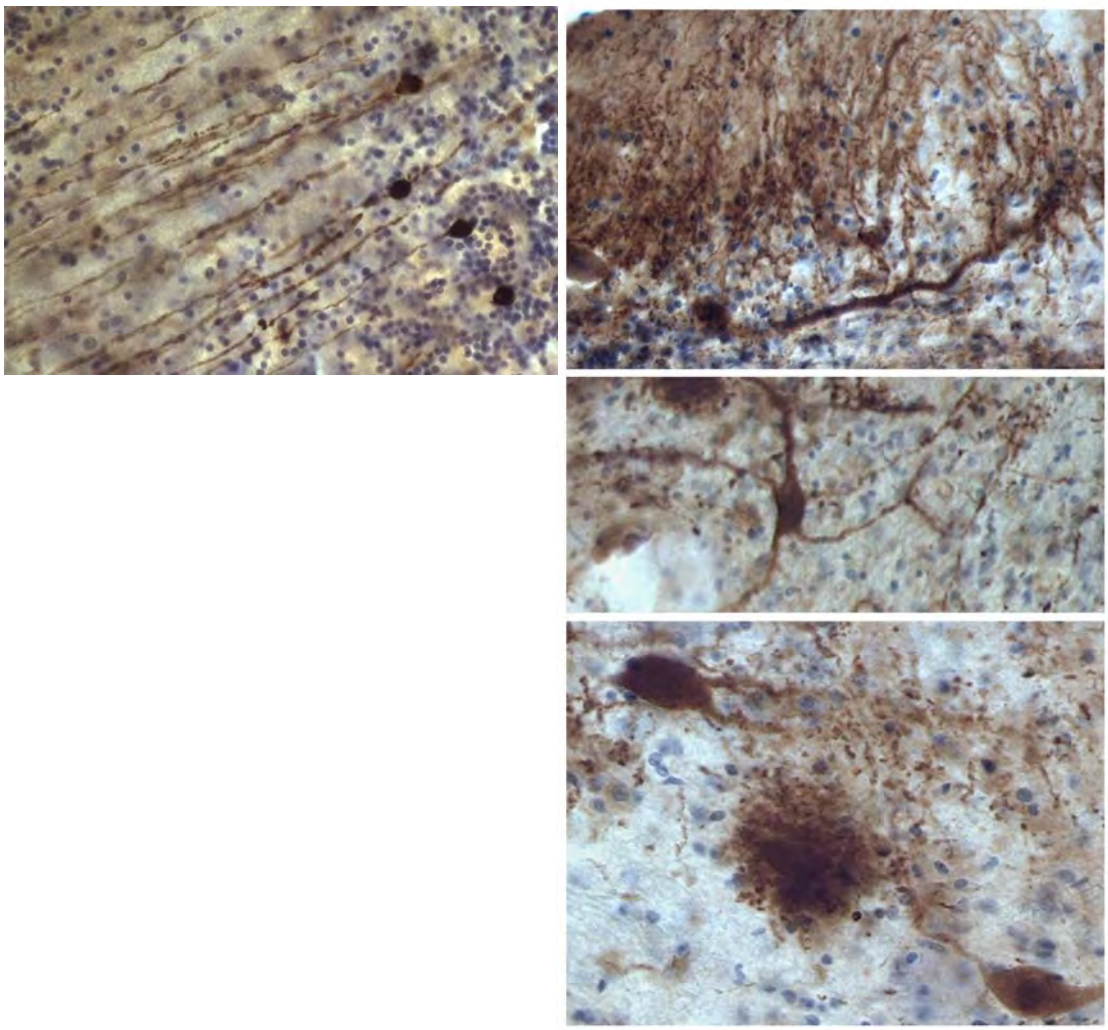
CHEMOARCHITECTURE (anti-calbindin mAb D-28)

Chemoarchitecture of not affected portion of the flocculus. In not affected portion of the flocculus the anti-calbindin mouse mAb D-28 revealed that Purkinje cells dendritic tree is the major factor determining structure of the molecular layer. Their axons penetrating granule cell layer and bundles of axons within the folia white matter contribute to cytoarchitecture of both, granule cell layer and white matter.

Pathology in the flocculus dysplastic area. Immunostaining for calbindin revealed striking deficit of Purkinje cells, and profound disorganization of the Purkinje cell layer, molecular layer, and white matter.



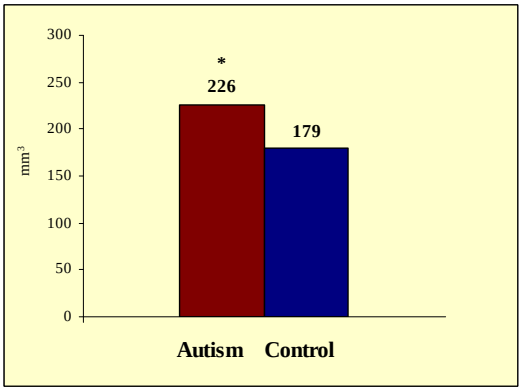
Defects of Purkinje cells. Four types of developmental abnormalities of Purkinje cells were found:
1. Lack of dendritic tree in cells with preserved axon.
2. Significant deficit of dendritic tree.
3. Deficit of dendrites, their abnormal morphology and spatial disorientation.
4. Presence of numerous very short spatially disoriented dendrites.



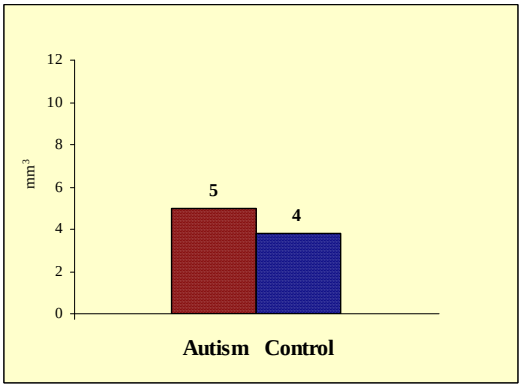
Inferior olive. CV-based morphometry revealed similar volume of the cell soma in autistic (4,019 μm^3) and control (4,012 μm^3) subjects. Calbindin immunostaining shows regional differences in distribution of calcium binding protein in inferior olive neurons both in autistic and control subjects.

MORPHOMETRY OF DYSPLASTIC AND NON-DYSPLASTIC FLOCCULUS

Flocculus volume. The mean volume of the flocculus in 12 autistic subjects was significantly more (226 mm^3, $p < 0.007$) than in 10 control subjects (179 mm^3).



Flocculus volume (mm^3)

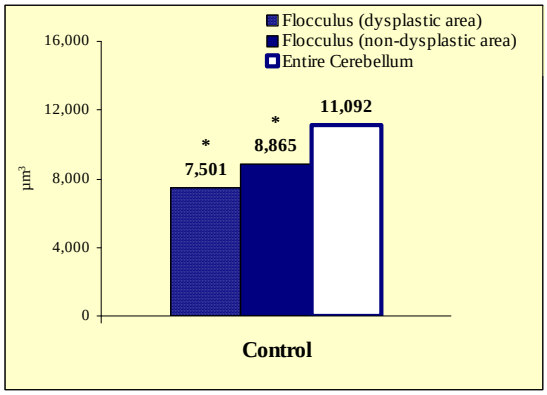


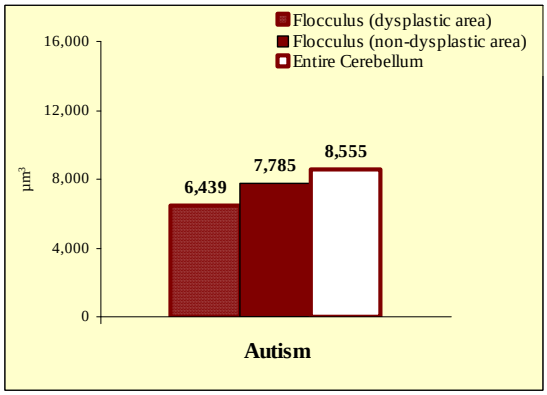
Floccular dysplasia volume (mm^3)

Floccular dysplasia volume. The average volume of dysplastic area in the flocculus of eight autistic subjects was 5.0 mm^3 and in two control subjects was 3.8 mm^3.

Interindividual differences in the volume of dysplastic area. The volume of the dysplastic area in the flocculus of 8 autistic subjects varied in a broad range from 0.2 to 12.8 mm^3. The volume of dysplasia in the flocculus of two control subjects was 0.8 mm^3 and 6.9 mm^3.

Purkinje cell volume in control subjects.
Purkinje cell volume in the not-dysplastic portion of the flocculus in 6 control subjects (8,865 μm^3) and dysplastic portion in one control case (7,501 μm^3) was significantly less than in the entire cerebellum of control subjects (11,092 μm^3, $p < 0.035$).
Purkinje cells volume in autistic subjects. The difference between the mean Purkinje cell volume in the not-dysplastic (7,785 μm^3) and dysplastic (6,439 μm^3) portion of the flocculus, and in the entire cerebellum (8,555 μm^3) of 6 autistic subjects was not significant**.**





Olivo-floccular circuit defects in autism. The olivo-floccular circuit is affected in 6/7 of autistic subjects by:
(a) focal floccular dysplasia with severe Purkinje cells deficit and abnormalities of Purkinje cells dendritic tree, and
(b) developmental inhibition of Purkinje cells growth in the flocculus and in the entire cerebellar cortex.
However, the second component of this circuit – the inferior olive – does not show changes in neuronal number and volume. Neuropathological study suggests that there are selective defects of the flocculus in the olivo-floccular circuit and indicate that floccular developmental abnormalities may have a major contribution to abnormal occulomotor activity, atypical gaze, altered movement perception and impairments in smooth pursuit detected in autistic subjects (Rosenhall et al, 1988, Scharre and Creedon 1992, Takarae et al, 2004).

CONCLUSIONS

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.

SUBPROJECT III. HYPOTHALAMIC NEURONS DEVELOPMENTAL DELAY IN AUTISTIC SUBJECTS

BACKGROUND

The hypothalamus accounts for only about 0.25% of the total brain weight, but neurons in two hypothalamic nuclei: nucleus paraventricularis (NPV) and nucleus supraopticus (NSO) are the major brain sources of two neurotransmitters, oxytocin and vasopressin.

It is hypothesized that genetic alterations in oxytocin and vasopressin neurotransmission may account for several features of autism. Oxytocin and vasopressin produced by hypothalamic neurons in the NSO and NPV have unique effects on normal expression of social behavior, attachment behaviors, formation and retention of social memory, communication, emotional response, thermoregulation, fluid and electrolyte balance, eating habits, energy metabolism, and immune response.

In autistic subjects, the level of oxytocin is reduced (Modahl et al 1998) but the level of C-terminal extended forms of oxytocin is increased, which suggests a deficit in oxytocin production and modifications of oxytocin processing (Green et al 2001). Patients with autism spectrum disorders show a significant reduction in repetitive behaviors following oxytocin infusion (Hollander et al 2003).

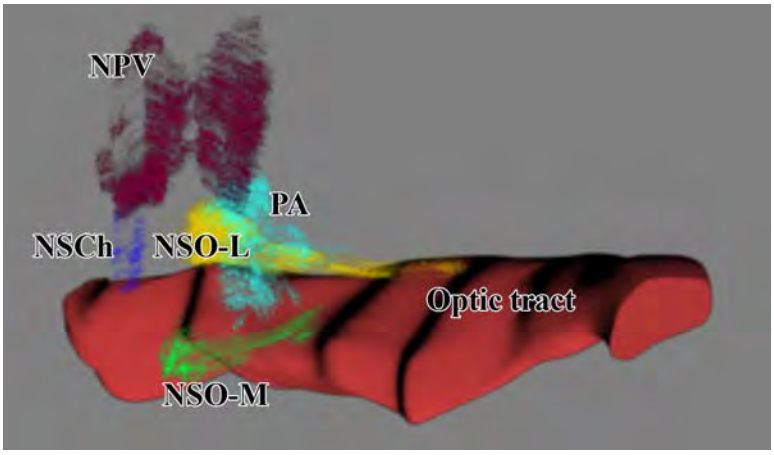
Hypothalamus abnormal structure/function may have a significant contribution to the autistic phenotype including social impairments, repetitive and stereotyped behaviors, anxiety, tantrums, self-injurious behavior and

AIMS. The aim of this study was:

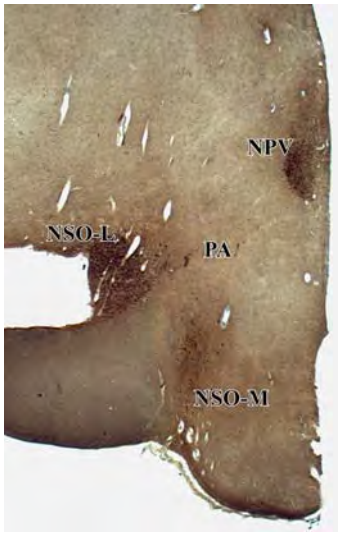
1. To detect and characterize modifications of development of neurons in the NSO and NPV, known as a major source for brain vasopressin and oxytocin.
2. To determine whether hypothalamic neurons developmental alterations may contribute to desynchronized development of neuronal circuits and behavioral abnormalities observed in autistic subjects.

MATERIAL. Brain hemispheres of 13 autistic and 14 control subjects 4 to 64 years of age were fixed in 10% formalin, dehydrated, embedded in celloidin, cut into 200 μm-thick serial sections and stained with cresyl violet. Due to incomplete preservation of the hypothalamic nuclei in the postmortem brain samples, the number of examined cases was reduced to 8 autistic (4 to 52 year old) and 10 control (4 to 32 year old) subjects. The fractionator method was used to determine the number of neurons, the Cavalieri method to estimate the volume of the NSO and NPV, and Nucleator method to determine the volume of neurons and neuronal nuclei (Microbrightfield, VT). The hypothalamus of 3 autistic and 6 control subjects was also examined in tissue embedded in polyethylene glycol (PEG), cut into 50 μm-thick sections, and immunostained with antibodies detecting oxytocin and vasopressin.

Hypothalamus anatomy. 3-D reconstruction illustrates the size and spatial distribution of the nucleus paraventricularis (NPV); medial and lateral portion of the nucleus supraopticus (NSO), preoptic area (PA), nucleus suprachiasmaticus (NSCh) and optic tract (OT).



Topography of hypothalamic nuclei. Free floating 50 μm-thick section was immunostained with antibody detecting vasopressin (23 year-old autistic subject). Nucleus supraopticus lateral and medial part (NSO-L and NSO-M), nucleus paraventricularis (NPV), and preoptic area (PA).



HYPOTHALAMIC NUCLEI CHEMOARCHITECTURE AND FUNCTION

Secretory activity. Hypothalamic neurons produce oxytocin, vasopressin, somatostatin, vasoactive intestinal peptide, dopamine, BDNF, neurotrophins, and hormone releasing factors. Oxytocin and vasopressin have a major contribution to behavior in normal conditions and behavioral alterations in autism.

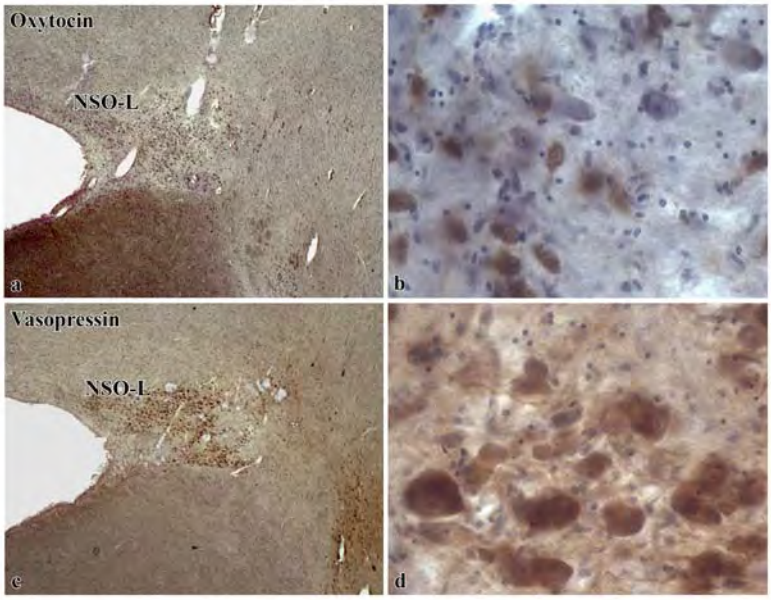
Vasopressin. Vasopressin enhances anxiety, aggressive behavior, stress levels, and consolidation of fear memory. Hypothalamic neurons innervate the entire cortical mantle and this innervation is essential for maintaining normal cortical arousal.

Oxytocin. Oxytoxin decreases anxiety and stress levels, facilitates social encounters, maternal care, reduces avoidance behavior, reduces activation of the amygdala, modulates fear processing.

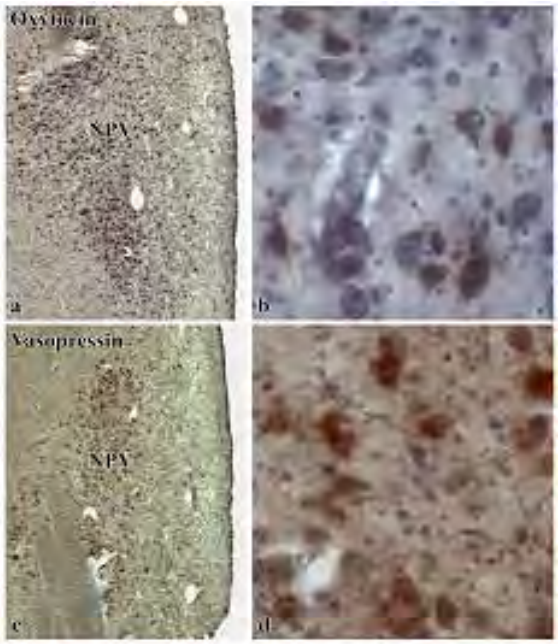
Number of vasopressin and oxytoxin producing neurons. The small size of highly specialized neuronal populations (40,000-94,000 vasopressin-positive neurons and only 9,000-12,000 oxytocin-positive neurons, makes this brain system extremely sensitive to genetic and epigenetic modifications resulting in clinical alterations.

NUCLEUS SUPRAOPTICUS

Small aggregates of neurons in the nucleus supraopticus (NSO) are oxytocin-positive (a and b), whereas almost all NSO neurons are vasopressin immunoreactive (c and d).



NUCLEUS PARAVENTRICULARIS



Low and high magnification of the nucleus paraventricularis (NPV) shows that only a small portion of neurons is immunopositive for oxytocin (a and b) and that a majority is vasopressin positive (c and d).

MORPHOMETRY

The average volume of the NSO of autistic (4.26 mm^3) and control (3.9 mm^3) subjects estimated in one brain hemisphere was comparable.

The NPV was larger than NSO, but the difference between the average volume of the NPV of autistic (6.28 mm^3) and control individuals (5.7 mm^3) also was not significant.

The average numerical density of neurons in the NSO of autistic subjects (31,990/mm^3) was similar to those estimated in control (33,156/mm^3) individuals.

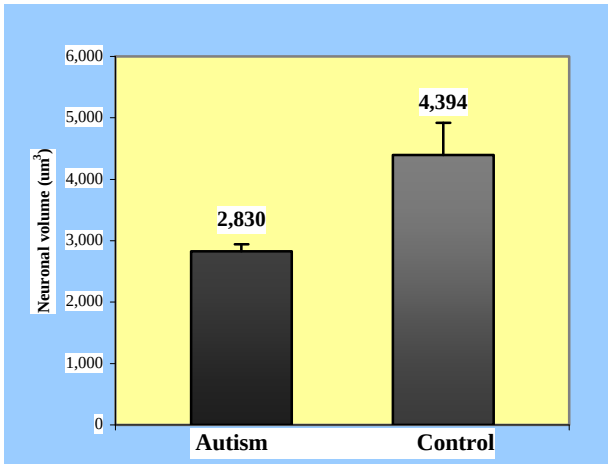
Numerical density of neurons in the NPV was two times more than in the NSO, in both autistic (62,550/mm^3) and control subjects (63,166/mm^3).
The difference between autistic and control subjects was not significant.
Total number of neurons in the NSO of autistic and control individuals was comparable (134,255 and 124,820), respectively.

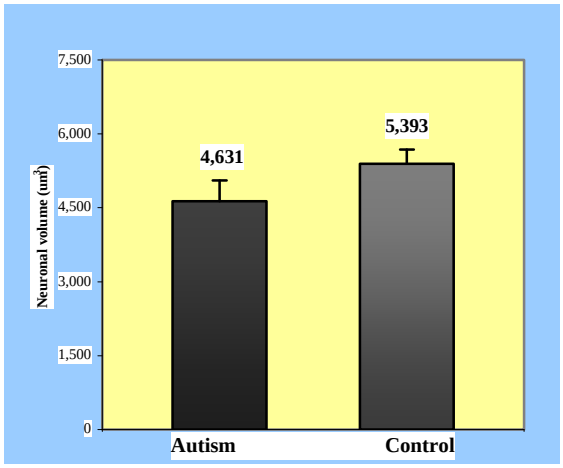
Total number of neurons in the NPV of both, autistic and control subjects was approximately 3x more than in NSO (405,519 and 367,921, respectively). The difference between these two groups was not significant.

THE MEAN VOLUME OF NEURONS IN THE N. SUPRAOPTICUS AND N. PARAVENTRICULARIS

The mean volume of neuron and neuron nucleus volume in the NSO and NPV were compared in eight autistic and ten control subjects. Ages of autistic subjects ranged from 4 to 52 years and of controls from 4 to 32 years. Stepwise regressions on autistic status, age, and their interaction showed neuronal size and nuclear size in the NSO to be significantly smaller in autistic subjects with age controlled.

NUCLEUS SUPRAOPTICUS

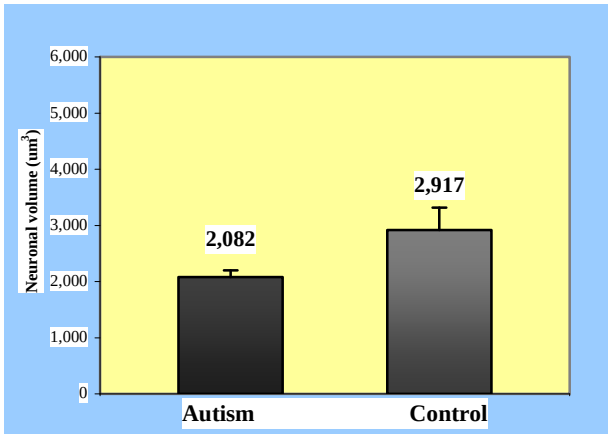


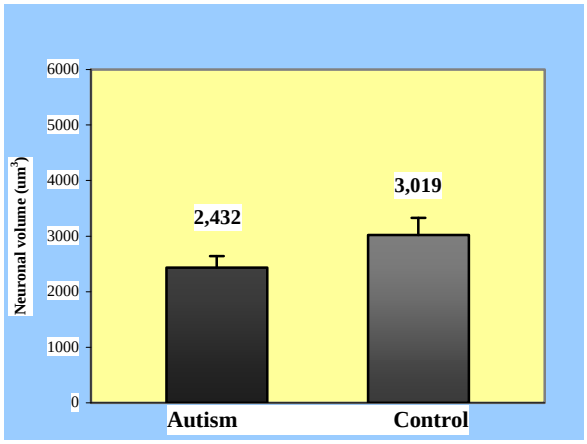


Mean neuronal size in NSO in autistic subjects 4 to 8 years of age was 35% less (2,830 μm^3) than in the control individuals (4,394 μm^3) (left panel). Mean neuronal volume in NSO in both groups increased with age, but the volume of neurons in autistic subjects more than 8 years of age was still not significantly (by 15%) less than in control individuals (right panel).

NUCLEUS PARAVENTRICULARIS

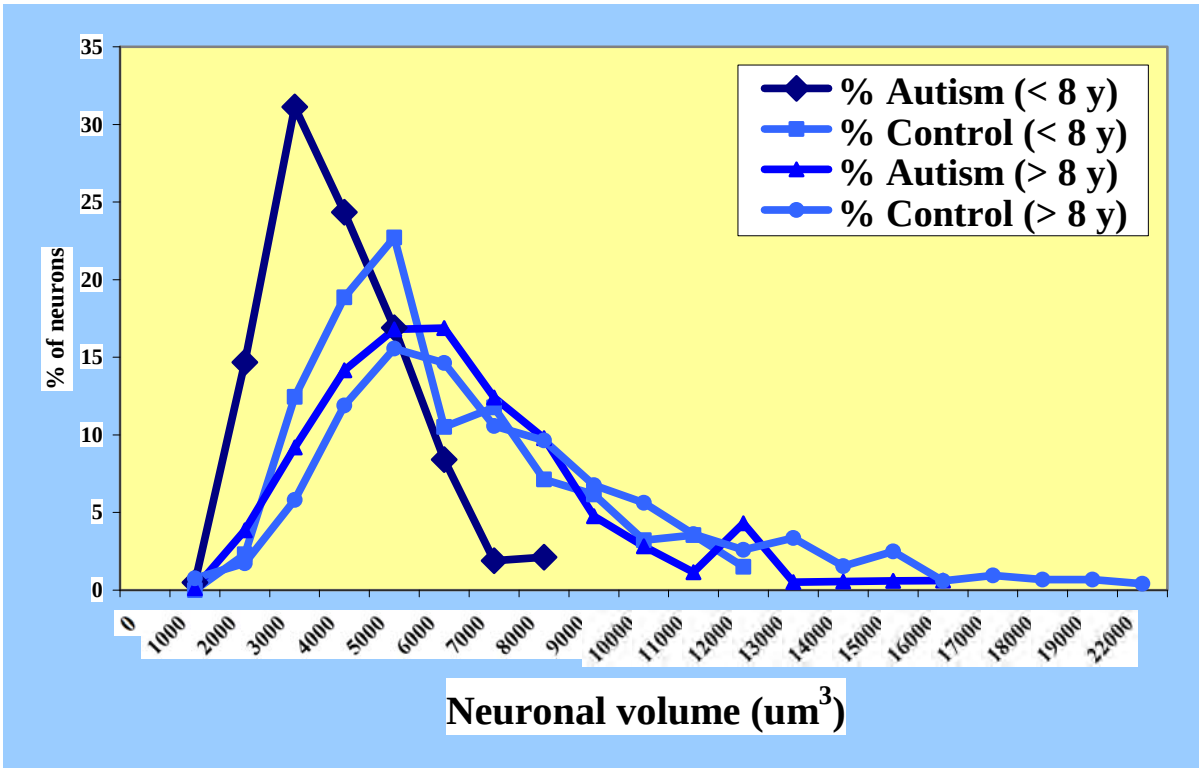
In the NPV of autistic subjects 4 to 8 years of age neurons were smaller by 29% (2,082 μm^3) than in control (2,917 μm^3) subjects (left panel). In individuals more than 8 years of age this difference was reduced to 20% (right panel).





Volume of neuron

Almost 70% of neurons in the NSO of autistic subjects less than 8 years of age are small neurons (less than 5,000 um^3), whereas in control subjects only 33% of neurons are small. In autistic subjects more than 8 years of age, the proportion between small and large neurons is reversed: 27% are small and 73% are large (more than 5,000 μm^3). This correction of neuronal size in autistic cohort results in a proportion closer to those observed in control individuals more than 8 years of age (20%/80%).



Neuronal nuclear volume in NSO. In the NSO of 4-8 years of age autistic subjects the mean cell nuclear volume (330 μm^3) was 42% less than in age matched control individuals (572 μm^3; $p < 0.002$). However, in subjects more than 8 years of age correction of nuclear volume reduced the difference between autistic and control subjects to not significant 8%.

Neuronal nuclear volume in NPV. In the NPV of 4-8 year of age autistic subjects the mean cell nuclear volume was 30% less than in control group (287 μm^3 and 405 μm^3 respectively, $p < 0.017$). In subjects more than 8 years of age 7% difference was not significant.

Conclusions

1. The study of two major components of the hypothalamus, the NSO and NPV, that are the major source of brain vasopressin and oxytocin, revealed that the total volume of these structures, numerical density and total number of neurons are not modified in autistic subjects.
2. However, application of unbiased stereological methods revealed that the neuron soma in autistic subjects 4 to 8 years of age is reduced in NSO and NPV by 35% and 29%, respectively. Neuronal nucleus volume was reduced by 42% and 30%, respectively.
3. In the NSO and NPV of autistic subjects, more than 8 years of age, a smaller volume was still present but the difference between autistic and control subjects was not significant.
4. Our studies of 24 brain structures suggest that developmental delay of growth of neurons in both, NSO and NPV is a component of desynchronized development of many brain structures, neuronal networks and interacting neuronal populations.
5. Detected abnormalities of hypothalamic neurons development may contribute to behavioral changes, including social interactions, anxiety, and aggression.

SUBPROJECT IV: ACCUMULATION OF AMYLOID-BETA PEPTIDES IN THE BRAIN OF CHILDREN WITH AUTISM

BACKGROUND

Enhanced beta-amyloid precursor protein (APP) processing with α-secretase, especially in subjects with aggressive behavior, was reported in an autistic cohort (Sokol et al, 2007). Bailey et al, (2008) detected a significant increase of secreted APP (sAPP-α) levels in the blood plasma in 60% of the autistic children. However, in contrast to the Sokol et al, study, there was no evidence of an association between elevated levels of sAPP-α and severity of aggression, and social or communication sub-scores. These studies supported the hypothesis that increased APP processing in the α-secretase pathway takes place in autism, and that the plasma levels of sAPP-α may be an early biomarker of at least a subgroup of children with autism (Bailey et al, 2008).

Our previous studies revealed that amino-terminally truncated Aβ is present in neurons in control subjects but intraneuronal Aβ immunoreactivity is not a predictor of brain amyloidosis-β or neurofibrillary degeneration (Wegiel et al, 2007). Our preliminary studies revealed enhanced accumulation of intracellular Aβ in the brain of subjects diagnosed with autism and with chromosome 15 duplication and autism. The majority of these subjects were also diagnosed as having seizures. Recent studies suggest a link between abnormal Aβ accumulation and epilepsy. Seizures are a prevalent phenotype in Fragile X syndrome (FXS), Alzheimer disease, and Down syndrome. Westmark et al (2010) experimental studies indicate that over-expression of APP/Aβ may contribute to seizures in these disorders.

AIMS

1. To detect difference between the patterns of brain region and cell-type specific distribution of intracellular Aβ in the brain of control subjects and subjects diagnosed with autism or idic15/autism.
2. To characterize pattern of intracellular Aβ distribution by confocal microscopy.
3. To detect the difference between properties of Aβ in the brain cortex and in cerebellum of control and autistic subjects.

MATERIAL AND METHODS

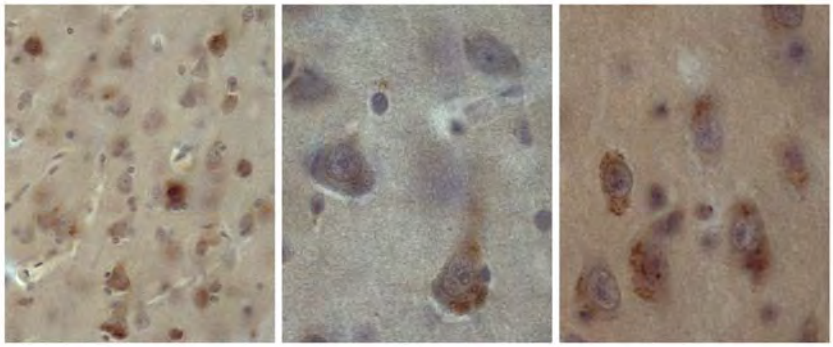
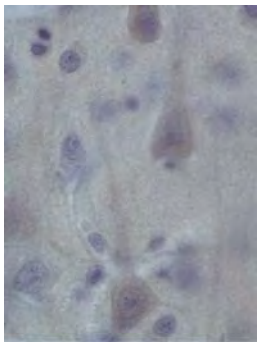
Formalin-fixed and frozen autopsy brain samples of 10 individuals from 9 to 56 years of age diagnosed with autism, and 12 control subjects from 13 to 59 years of age, were used in this study. Diagnosis of autism was confirmed by Autism Diagnostic Interview – Revised (ADI-R).

Formalin-fixed brain hemispheres were dehydrated in ascending ETOH concentrations and embedded in polyethylene glycol (PEG). Aβ was immunolabelled in free-floating sections using mAb 4G8 detecting Aβ17-24 and mAb 6E10 specific for the 1-16 aa Aβ sequence.

Confocal microscopy. The sections were double immunostained using mAb 4G8 and rabbit antisera against autophagic vacuole marker Lamp1 (Abgent) and lysosomal marker cathepsin D (Calbiochem). Secondary antibodies were the respective species-specific antisera conjugated with Alexa 488 or Alexa 555 (Invitrogen).

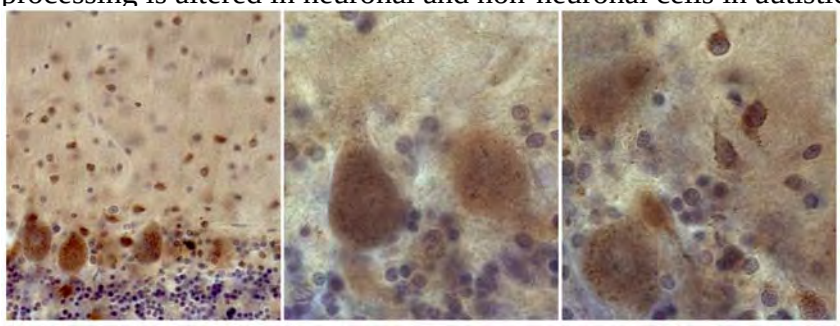
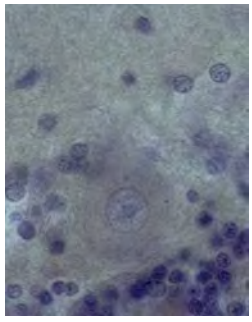
Immunoblotting. Aβ was detected in samples of frozen frontal and temporal cortex, cerebellar cortex and dentate nucleus. Aβ40 and Aβ42 was detected with affinity purified rabbit antibodies R162 and R226, specific for the respective Aβ C-terminal aminoacids. Sequential centrifugation of brain lysates at 1,000g for 5 minutes, 16,000 g for 10 min, and 100,000 g for 30 min was used to pellet large and small cellular deposits, and cell membranes, respectively.

Idic15/autism, 11y (mAb 4G8 detecting Aβ17-24aa and mAb 6E10 detecting Aβ1-17aa)
Mapping of Aβ17-24 aa in the brain of male diagnosed with idic15, autism, and intractable epilepsy, whose sudden unexpected death at age of 11 years was seizure related reveals brain region and cell type specific pattern of abnormal Aβ accumulation in the cytoplasm of neurons and glial cells (Aβ is 4G8-positive and 6E10 negative; α-secretase product).
Neocortex. About 40% of neurons in the frontal and temporal cortex accumulate Aβ in clusters of irregular in shape and size immunoreactive granules.

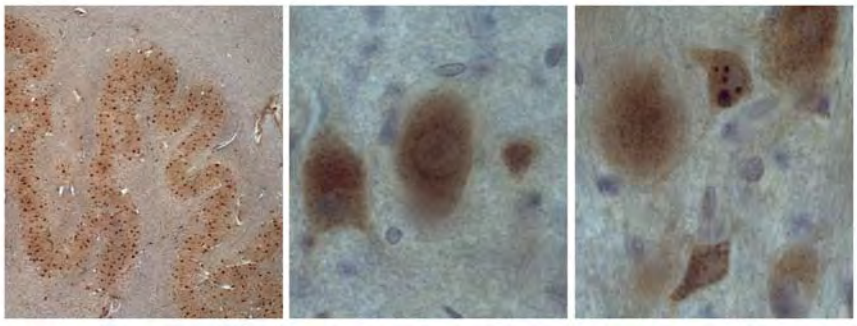
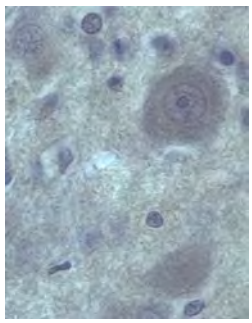
4G8 **6E10**

Cerebellar cortex. Accumulation of small Aβ granules in the soma of almost all Purkinje cells and in numerous glial cells in the molecular layer of cerebellar cortex suggests APP processing is altered in neuronal and non-neuronal cells in autistic subjects.

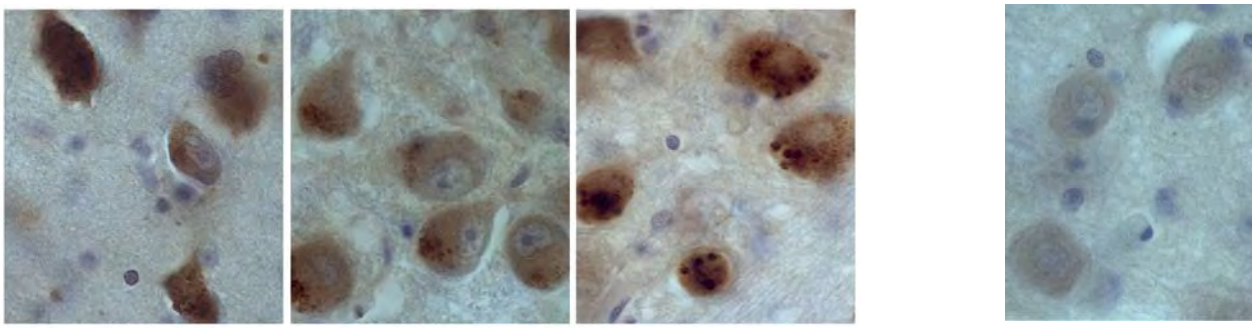
4G8 **6E10**

Dentate nucleus. Deposition of numerous small Aβ-positive granules in large neurons and a few polymorphic large granules in small neurons in the dentate gyrus, reflect cell type-specific APP processing and Aβ trafficking within one cerebellar structure.

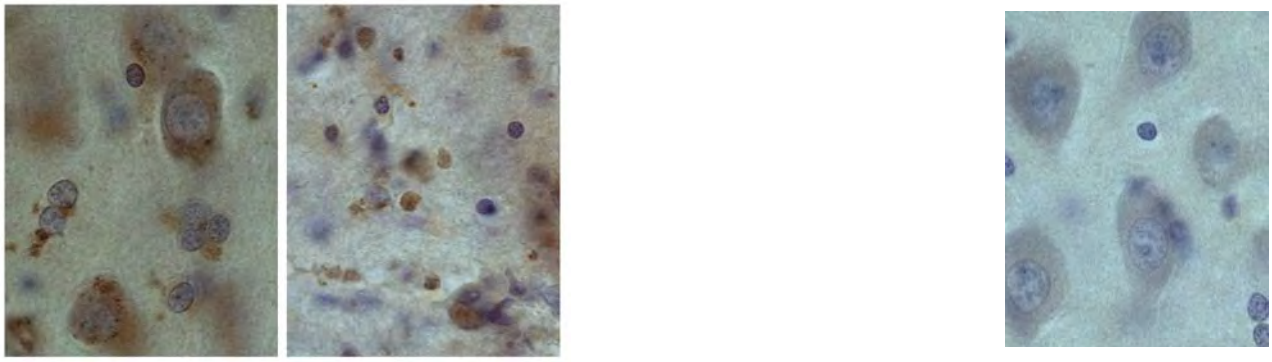
4G8 **6E10**

Amygdala, lateral geniculate body, and thalamus. Large amount of Aβ-positive granules in perinuclear region in almost all neurons in the amygdala and lateral geniculate body, and several large granules in the perinuclear region in neurons in the reticular nucleus of the thalamus illustrate differences between APP processing and Aβ trafficking in different brain structures/neuronal networks.



4G8 **6E10**

Hippocampus. In the cornu Ammonis, Aβ-accumulates not only in a majority of neurons in the cell soma but also in astrocytes, especially often in the CA4 sector. In the perivascular space, Aβ-positive deposits are observed in the cytoplasm of astrocytes and macrophage-like cells, and in extracellular space as large 4G8-positive aggregates.



4G8

Control subjects mAb 4G8 (Aβ17-24)

Mapping of Aβ in the brain of control subject reveals weak immunoreactivity in a few cortical, subcortical and cerebellar neurons, and no reaction in glial cells.

Neocortex. Few fine granular Aβ-positive deposits are detected in only 2-3% of neurons in the frontal and temporal cortex.

Cerebellar cortex. Aβ-positive granules are present in small cells in the Purkinje cell layer but almost all Purkinje cells and glial cells in the molecular layer are 4G8-negative.

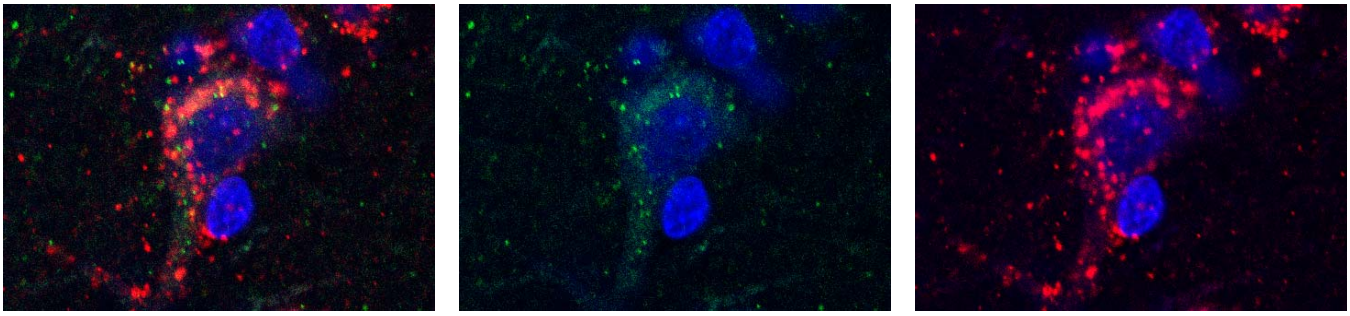
Dentate nucleus. About 40% of neurons in the dentate nucleus show weak Aβ-immunoreactivity. Approximately 40% of small neurons contain large 4G8-positive granules.

Amygdala, lateral geniculate body, and thalamus. A very few neurons show weak Aβ-immunoreactivity in the amygdala and lateral geniculate body, but some neurons in the reticular thalamic nucleus contain several large granules in the perinuclear region.

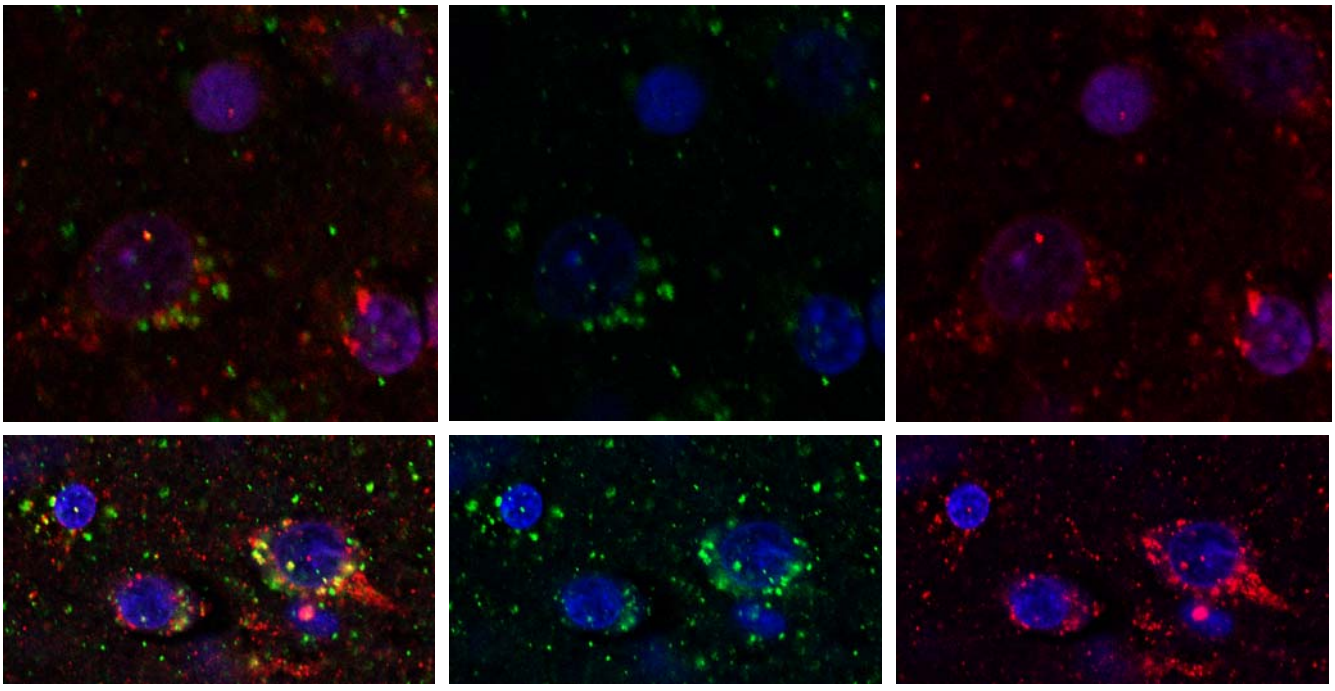
Hippocampus. A very few neurons in the cornu Ammonis, including CA4 are Aβ-immunopositive. Glial cells are Aβ-negative.

Intracellular localization of Aβ (mAb 4G8)
(Confocal microscopy, frontal cortex, 10 year-old idic15/autistic subject)

In neurons with a few mAb 4G8-immunoreactive deposits (green) co-localization of Aβ with the lysosomal marker cathepsin D (red) is observed. However, in neurons with numerous 4G8-positive deposits only a fraction of Aβ is detected in lysosomes.



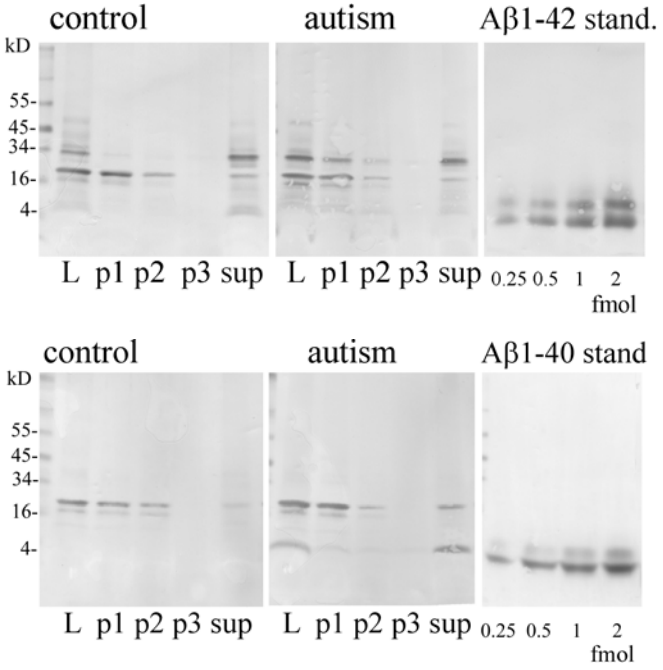
Neurons and glia contain Aβ deposits (green) located both in and out of Lamp1-positive autophagic vacuoles (red). Only a minor portion of intracellular Aβ is detected in autofluorescent lipofuscin deposits.

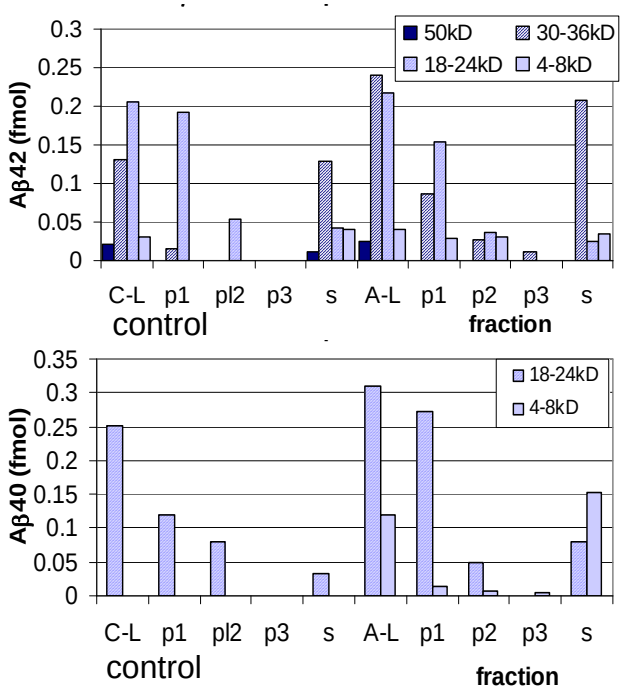


Amount and properties of Aβ in cerebral cortex and cerebellum of autistic and control subjects

Characterization of Aβ40 and Aβ42 in full lysates (L) and in cellular deposits obtained by sequential centrifugation of homogenates of the dentate nucleus of 20 year-old autistic and 27 year-old control subject. Pellets were obtained at: 1,000 g for 5 min (p1); 16,000 g for 10 min (p2). Pellet (p3) and supernatant (sup) were obtained at 100,000 g for 30 min.

- Most of Aβ42 is present in 16-24 kD complexes detected in large subcellular structures sedimented at 1,000g (p1).
- Aβ42 monmomers and the 30-34 kD complexes are mainly present in the cytosol (sup).
- Most of Aβ40 is present in the 16-24 kD complexes which are detected in large subcellular structures (p1).





Aβ42 in brain lysates in autistic and control subjects. In all examined brain regions Aβ42 is detected in the monomeric form, but majority of Aβ42 is detected in complexes of different molecular sizes. In autism, all the tested structures contain significantly more of 30-34 kD Aβ42 complexes than in controls. These complexes are present mainly in the cytosolic fraction.

Lysates of the cerebellar cortex and dentate nucleus of autistic subjects contain more of 30-34 and 16-24 kD Aβ42 complexes than control samples. The 16-24 kD Aβ42 complexes are associated mainly with large subcellular structures.

CONCLUSIONS

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 structure, suggests that enhanced intracellular accumulation of Aβ in autism result from different pathways of APP processing and Aβ deposition.
5. Detection of increased levels of Aβ complexes in the soluble and insoluble form in 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.

KEY RESEARCH ACCOMPLISHMENTS

Project 2 integrates three major research strategies including the study of the contribution of:
1. Qualitative developmental abnormalities to the autistic phenotype.
2. Quantitative developmental abnormalities to the autistic phenotype.
3. Developmental neuronal metabolic alterations to the clinical phenotype of autism

1: Contribution of qualitative developmental abnormalities to the autistic phenotype.
Cooperation with Project 1 provided historically the most complete characteristics of qualitative neuropathological changes in the largest cohort of autistic subjects examined in one standard (23 cases) and characteristics of neuropathological changes in the brain of individuals diagnosed with autism dup(15) (9 cases) and age-matched controls (24 cases).

(a) The product of this neuropathological qualitative studies are characteristics of the type, topography, severity and prevalence of developmental brain abnormalities in autism with an unknown etiology and autism caused by maternal origin dup(15) (Wegiel et al 2010a and b).
(b) This study revealed failure of mechanisms that control: neuronal proliferation, migration, and brain region specific cytoarchitecture. The effects are heterotopias, dysplastic changes and subependymal nodular dysplasia.
(c) These focal abnormalities have a strong contribution to an early onset of epilepsy, functional regression and an increased risk of Sudden Unexpected Death in Epilepsy (SUDEP). They are 2.4 times more frequent in autism dup(15) than in idiopathic autism.
(d) The study of the autism dup(15) cohort revealed that in majority of affected subjects autism is associated with microcephaly, whereas the study of idiopathic autism cohort indicated that autism is associated with macrocephaly. It indicates that failure of mechanisms controlling brain growth results in inhibition or overgrowth that may contribute to autism.

The applied research strategy indicates that neuropathological studies are able to identify both focal developmental alterations and global defects of brain and brain subdivisions growth, and their contribution to specific functional defects observed in autism.

2. Contribution of quantitative developmental abnormalities to the autistic phenotype.

This study integrates (i) localized models of defective development of neurons with (ii) more complex models of altered neuronal circuits into (iii) a global model of brain development desynchronization.

The following structures and their anatomical subdivisions were examined:

(a) Limbic system involved in emotions, social behavior (amygdala), memory processing (entorhinal cortex) and storage (cornu Ammonis);
(b) Several subdivisions of the striatal networks involved with stereotypic behaviors (caudate, putamen, globus pallidus) and reward (nucleus accumbens);
(c) The thalamus, as a key component of networks implicated in attention, memory, language, and emotional processing;
(d) The cerebellum (Purkinje cells and dentate nucleus) involved in language and motor functions.
(e) Purkinje neurons, unipolar brush neurons, and granule cells in the cerebellar flocculus involved in abnormal oculomotor activity, abnormal gaze, and poor eye contact of autistic subjects.
(f) Claustrum (two subdivisions) integrating function of several brain modalities with major contribution to cognition.
(g) Substantia nigra was selected as a source of dopamine controlling motor, reward and other systems.

(h) Nucleus basalis of Maynert (four subdivisions CH1-4)) was selected as the source of brain acetylcholine.
(i) Hypothalamus (N. supraopticus and n. paraventricularis) was selected as the major source of oxytocin and vasopressin regulating social memory and interactions, communication, ritualistic behaviors, aggression and anxiety, sleep and other features of autism.

Project 2 identified quantitative differences in neuronal development with striking delay of neuronal growth in 3-8 years of age autistic children. However, the range of delay was neuron-type specific. This study identified desynchronization of development of neurons, neuronal circuits and neurotransmitter systems as the major contributor to the autistic phenotype. 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.

3. Contribution of developmental neuronal metabolic alterations to the clinical phenotype of autism.

(a) 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.
(b) Aβ accumulation in neuronal cathepsin D- and Lamp1-positive lysosomes and lipofuscin, indicates that enhanced α-secretase processing is paralleled by an enhanced proteolytic activity.
(c) 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.
(d) The accumulation of $A\beta_{17\text{-}40/42}$ in the astrocytes of some autistic children and adults, and in the plaque perimeter in all three plaque-positive subjects may indicate that the astrocyte cytoplasmic Aβ load reflects a local enhancement extracellular Aβ levels of the neuronal origin and an astrocyte contribution to Aβ clearance.
(e) The higher prevalence of Aβ alterations, early onset of intractable seizures, and a high risk of SUDEP in autistic subjects with dup(15) as compared to subjects with idiopathic autism supports the concept of mechanistic and functional links between autism and alterations of APP processing, neuronal and glial Aβ accumulation, and diffuse plaque formation.

REPORTABLE OUTCOMES

1. The neurobiological and neuropathological background of this Program Project is summarized in our book chapter:
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. Results of neuropathological evaluation of brains of 13 autistic and 14 control subjects are summarized in our paper published in *Acta Neuropathologica*:
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. The neuropathology of autism: defects of neurogenesis and neuronal migration, and dysplastic changes. Acta Neuropathologica, 2010, 119,755-770

International Meeting for Autism Research,
(IMFAR 2011, San Diego, CA, May 12-14, 2011)

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. 10th Annual International Meeting for Autism Research, San Diego, CA, May 12-14, 2011 (Oral presentation).

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 J. 10th Annual International Meeting for Autism Research, San Diego, CA, May 12-14, 2011 (Poster)

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.10th Annual International Meeting for Autism Research, San Diego, CA, May 12-14, 2011. (Poster)

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. 10th Annual International Meeting for Autism Research, San Diego, CA, May 12-14, 2011 (Poster)

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. Annual International Meeting for Autism Research, San Diego, CA, May 12-14, 2011 (Poster)

Manuscripts ready for submission (See Appendix):

Abnormal intracellular and extracellular Aβ deposition in idiopathic and dup15 autism. Wegiel J, Frackowiak J, Mazur Kolecka B, Schanen NC, Cook EH Jr, Sigman M, WT Brown, 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, Flory M, Wisniewski T. J Neurosc.

Differences between the pattern of developmental abnormalities in autism associated with duplications 15q11.2q13 and idiopathic autism. Wegiel J, Schanen NC, Cook EH Jr, Sigman M, WT Brown, 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. Am J Hum Genet.

Manuscripts in preparation for submission:

Developmental delay and desynchronization of neuronal growth in autism. Wegiel J, WT Brown, Kuchna I, Nowicki K, Ma SY, Wegiel J, Imaki H, Chauhan A, Chauhan V, Cohen IL, London E, Flory M, Wisniewski T. Am J Neuropath Exp Neurol

Clinical implications of the olivo-floccular circuitry developmental defects in autism. Wegiel J, WT Brown, Kuchna I, Nowicki K, Ma SY, Wegiel J, Imaki H, Chauhan A, Chauhan V, Cohen IL, London E, Flory M, Wisniewski T.

CONCLUSIONS

1. Significant similarities of developmental alterations of neuronal proliferation, migration, and cytoarchitecture in idiopathic autism (with unknown etiology) and autism caused by dup(15) indicate that, in part, developmental defects are caused by similar/same mechanisms regardless of the etiological factor. However, (a) the absence of cortical dysplasia, (b) presence of a several fold more frequent pathology in the dentate gyrus, (c) more intraneuronal Aβ in neurons, (d) a very high prevalence of early onset of seizures in individuals with autism dup(15) compared to the idiopathic autism subjects indicate that the etiology has a specific contribution to structural, biochemical and functional changes in autism.
2. Striking brain region specific delays of neuronal growth in children 3-8 years of age, indicate that autism is caused by failure of mechanisms controlling neuron and brain growth.
3. Regional dysregulation results in desynchronization of growth of interacting neurons, neuronal circuits, and neurotransmitter systems.
4. These developmental defects appear to be the major contributor to social and communication deficits, ritualistic behavior and intellectual deficits.
5. Enhanced APP processing with α- and γ-secretases, leading to enhanced accumulation of Aβ in neuronal cytoplasm observed in the majority of autistic subjects, including children, is an early sign of an altered non-amyloidogenic pathway of APP processing.
6. Early onset of diffuse plaques (at age of 39, 51 and 52 yrs) indicates increasing risk of early activation of amyloidogenic pathway of APP processing in autism with all consequences of intracellular and extracellular accumulation of toxic, oligomerized and fibrillized Aβ.
7. This study indicates that autism has age-specific manifestations of altered mechanisms, structural and functional modifications.

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Appendices

Abnormal intracellular and extracellular Aβ deposition in idiopathic and dup 15 autism

Abreviated title: Aβ accumulation in autism

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Summary

Amyloid ß (Aβ), a product of the proteolytic cleavage of the amyloid β precursor protein (APP), accumulates in control subjects in neuronal cytoplasm in a cell-type specific amounts. 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 chromosome 15 duplication (dup15) and autism, including children. This suggests there exists an early alteration of APP processing with α-secretase. Aβ accumulation in neuronal 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 autistic subjects, 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 unexplained death in epilepsy (SUDEP) in autistic subjects with dup(15) compared to subjects with idiopathic autism supports the concept of there being mechanistic and functional links between autism and alterations of APP processing, neuronal and glial Aβ accumulation, and diffuse plaque formation.

Key words: autism, chromosome 15 duplication, epilepsy, intracellular amyloid beta, diffuse plaques.

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 (American Psychiatric Association, 2000). Autism is often diagnosed in subjects with genetic disorders, including maternal duplication of 15q11q13 (dup(15); 69%, Rineer et al 1998, Simon et al 2000), fragile X syndrome (FXS) (15-28%, Hagerman 2002), and Down syndrome (DS) (at least 7%, Kent et al 1999).

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, 2011, Bailey et al 2008, Westmark and Malter 2007).

The proteolytic cleavage of APP by membrane associated secretases releases several Aβ peptides possessing heterogeneous amino- and carboxyl-terminal residues including: $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ as products of β- and γ-secretases (amyloidogenic pathway); $A\beta_{17\text{-}40/42}$, as a product of α- and γ-secretases (p3 peptide, non-amyloidogenic pathway) (Iversen et al 1995, Selkoe 2001); and $A\beta_{pE3}$ as a product of N-terminal truncation of full length Aβ peptide by aminopeptidase A and pyroglutamate modification (Sevalle 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 Wirths 2010).

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 (Bailey et al 2008). Enhanced APP processing by α-secretase, is especially prominent in autistic subjects with aggressive behavior (Sokol et al, 2006, Ray et al 2011). 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 $A\beta_{42}$ (Westmark and Malter 2007). Sokol et al (2011) 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 of autistic children and macrocephaly in FXS.

Experimental studies in fmr1 KO mice (Westmark et al 2010) suggest that over-expression of APP/Aβ may contribute to the seizures observed in autism (Tuchman and Rapin, 2002) and FXS (Hagerman 2002) and that both the over- and under-expression of APP and its metabolites, increases incidence of seizures (Moechars et al 1996, Westmark et al 2007, 2008, 2010).

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 (Wegiel et al 2007). One may hypothesize that increased levels of sAPP-α in blood plasma (Sokol et al, 2006, Ray et al 2011, Bailey et al 2008) reflect an 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 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 ($A\beta_{17-40/42}$); (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.

Materials and methods

Material. The brains studied were from 9 individuals diagnosed with dup(15) with ages 9 to 39 years (5 males and 4 females), 11 subjects with idiopathic autismwith ages 2 to 52 years (10 males and 1 female), and 8 control subjects with ages 8 to 47 years (4 males and 4 females) (**Table 1**). Medical records were obtained following consent for release of information from the subjects' legal 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. Clinical and genetic studies were performed as described previously (Wegiel et al, submitted). 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 (Lord et al 1994).

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 (STRP) from chromosome 15, Southern blot analysis of dosage with 5-12 probes, measurement of the methylation state at *SNRPN exon α*, as described (Mann et al, 2004), and array comparative genomic hybridization (CGH) (Wang et al, 2004). Duplication morphology was confirmed by fluorescent in situ hybridization (Mann et al, 2004).

In eight cases, tetrasomy, and in one case, hexasomy of the Prader-Willi/Angelman syndrome critical regions (PWACR), 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, 7/9 subjects (78%) were diagnosed with autism spectrum disorder (ASD), and seven had seizures. In six cases (67%) sudden unexplained death in epilepsy (SUDEP) was

reported. In the idiopathic autism cohort, two subjects (8 yr old male, HSB4640 and 52 yr old male, BB13760), were diagnosed with atypical autism or high functioning autism. In other cases the clinical diagnosis of autism was confirmed with ADI-R.

Tissue preservation for neuropathology. 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 (Wegiel et al 2010, Wegiel et al submitted). The mean postmortem interval (PMI) varied from 23.9 hours in the dup(15) cohort, to 19.6 hours in the idiopathic autism cohort and 15.0 hours in the control group. One brain hemisphere from each subject was fixed in 10% buffered formalin for a period of time ranging from 6 weeks to several months, dehydrated in a graded series of ethanol, infiltrated and embedded with polyethylene glycol (PEG) (Merck) (Iqbal et al, 1993) and stored at 4ºC. 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; Wegiel et al 2010) and cut alternatively into 200 and 50 μm-thick serial sections.

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) the Aβ distribution in cells in the cerebral cortex, subcortical structures, cerebellum, and brainstem; (b) the Aβ peptide properties; and (c) Aβ distribution in lysosomes and lipofuscin (Table 2).

Monoclonal antibodies (mAbs), 6E10 and 6F/3D were used to characterize the N-terminal portion of Aβ. mAb 6E10 recognizes an epitope in residues 4-13 of Aβ (Signet Laboratories, 1:10,000) [Kim et al 1990, Miller et al 2003]. mAb 6F/3D recognizes an epitope in residues 8-17 of Aβ (Novocastra Laboratories LTD). The middle portion of Aβ was detected with mAb 4G8, which recognizes an epitope in residues 17-24 of Aβ [Kim et al 1988]. The carboxyl terminus of Aβ was characterized with rabbit monoclonal antibodies Rabm38, Rabm40 and Rabm42 detecting $Aβ_{-38}$, $Aβ_{-40}$, $Aβ_{-42}$, respectively (Miller et al 2011). Stern et al (1989) have shown that full length APP is very sensitive to fixation methods and that it's immunogenicity is easily lost; therefore, immunostainings for Aβ are specific and do not detect APP in formalin fixed human postmortem brain tissue samples. Furthermore antibodies to the carboxyl terminus of Aβ do not recognize APP and are specific for Aβ peptides (Gouras et al 2010).

To detect intracellular Aβ peptides and amyloid in plaques, free-floating sections were treated with 70% formic acid for 20 min (Kitamoto et al 1987). The endogenous peroxidase in the sections was 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 min to block nonspecific binding. The 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.

Double immunostaining for Aβ (mAb4G8) and for astrocytes (GFAP) was carried out to confirm the presence of Aβ in astrocytes. Confocal microscopy was conducted to detect Aβ localized in neuronal cytoplasmic organelles. To detect Aβ, brain sections were treated with 70% formic acid for 20 minutes, washed in PBS 2x 10 min and double immunostained using mAb 4G8 and lysosomal marker cathepsin D (Calbiochem) or a rabbit polyclonal antibody against lysosomal associated membrane protein (LAMP1) (Abgent). 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 confirmed as previously described (Frackowiak et al 2003). Images were generated using a Nikon C1 confocal microscope system with EZC1 image analysis software.

RESULTS

Mapping of increased intraneuronal Aβ accumulation in autistic subjects. In most all subjects with dup15/autism and the majority of individuals with idiopathic autism, intraneuronal Aβ immunoreactivity was observed in more neurons and immunoreactivity was stronger than in the control subjects (Fig. 1). Five sub-patterns of intracellular Aβ deposition were distinguished.

In the brain of the autistic subjects, the strongest and most consistent Aβ immunoreactivity was observed in three structures: the dentate nucleus in the cerebellum, the inferior olive in the brainstem and the lateral geniculate body, with almost all neurons positive for Aβ and with a larger amount of immunoreactive granular material per cell than seen in other brain structures. A similar distribution, but with much smaller amounts of Aβ, was also observed in the control brains.

A moderate amount of Aβ immunopositive material was accumulated in almost all of the neurons in the amygdala, thalamus, globus pallidus, and the CA4, CA3 and CA2 sectors of the cornu Ammonis of the autistic individuals. In the control brains, more neurons were negative or contained only a small amount of Aβ.

A moderate amount of Aβ was also observed in the cerebral cortex of the autistic subjects, but cortical deposits showed significant region and layer-specific differences. More Aβ was observed in the pyramidal neurons and in the 6th layer than in the granule neurons. Moreover, the neuronal amyloid load was strikingly different in individual neurons. In the majority of autistic subjects, the percentage of pyramidal neurons with a heavy Aβ load reached 60-80%. The percentage of amyloid rich neurons was much lower in the control subjects.

In the control subjects, a small amount of Aβ appeared in some small neurons in the caudate/putamen, n accumbens, CA1 sector, and granule cell layer of the dentate gyrus. In a majority of the autistic subjects, each of these structures had more Aβ-positive neurons and those neurons revealed much more Aβ immunoreactivity than in the control brains. In the majority of autistic subjects, almost all neurons in granule cell layer of the dentate gyrus were Aβ-positive and amount of immunoreactive material was several fold more than in control cases.

The morphology of the intracellular deposits of Aβ-positive material was cell-type specific. In Purkinje cells there were granular deposits accumulated in the cell body. In the dentate nucleus, large neurons accumulated fine-granular material, whereas small neurons accumulated a few large-moderate size Aβ-positive vacuoles. Neurons in the reticulate nucleus in the thalamus contained a mixture of fine-granular material and large 4G8-positive granules. Cortical pyramidal neurons showed significant heterogeneity of intraneuronal deposits with a mixture of fine-granular material and several-times larger 4G8-positive granules.

Aβ in glial cells. Astrocytes and microglia in the control brains were Aβ-negative. Enhanced neuronal Aβ accumulation in the brains of individuals with autism was usually associated with Aβ accumulation in the astrocytes cytoplasm, and in some microglial cells. Confocal microscopy confirmed the Aβ accumulation in astrocytes (Fig. 2). 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 in the cerebellar cortex border zone between granule and molecular layers). The less common form was Aβ immunoreactive granular material deposition in the entire cell 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 several-fold increase of the number of astrocytes, all A β-positive (Fig. 2a), clustering of astrocytes in groups of 3-10 cells (Fig. 2b), numerous mitoses as a sign of astrocyte proliferation (Fig. 2c, d) and astrocyte death resulting in deposition of extracellular remnants of Aβ aggregates (Fig. e) 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.

The difference between the pattern of intraneuronal Aβ accumulation in the autistic and the control age matched brains can be defined as (a) an age-independent enhancement of Aβ immunoreactivity above the control level in almost all neuronal populations, (b) an accumulation of strikingly large amounts of immunopositive material in neurons, which were almost immunonegative in the control brains (granule neurons in the dentate gyrus), and (c) the appearance of Aβ in astrocytes, their proliferation, and death resulting in extracellular Aβ deposition. This global pattern was modified in individual cases of autism or autism associated with dup(15) and suggests it is a reflection typical for the etiological and clinical heterogeneity of autism. These changes were detected in the majority of subjects diagnosed with idiopathic autism and autism/dup(15) but were usually more pronounced in autism associated with dup(15).

Immuno characterization of intraneuronal Aβ. Intraneuronal Aβ deposits revealed striking neuron-type specific differences in the amount, morphology and cytoplasmic distribution; however, they had the same immunoproperties. They revealed no reaction or traces of reaction with mAb 6E10 (Fig. 1) and 6F/3D (not shown). Positive reactions with mAb 4G8 and Rabm38, Rabm40 and Rabm42 indicated that the cytoplasmic deposits in the neurons of control subjects and individuals with idiopathic autism and autism/dup(15) were almost exclusively $A\beta_{17\text{-}40/42}$, and that they were the product of α and γ secretases.

Diffuse plaques distribution and immunoproperties. 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 yr old female who was also diagnosed also with autism, 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. 3). Plaques were also found in the brain of two individuals diagnosed with idiopathic autism, including a 51 yr old subject , who had only had one grand mal seizure, and a 52 yr old individual whose records do not contain information about epilepsy or brain trauma (Fig. 4). In both brains, the postmortem examination revealed numerous plaques within the entire cortical ribbon, in the amygdala, thalamus, and the subiculum.

In all three cases, plaques stained with thioflavin S did not reveal fluorescence (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, revealed full-length $A\beta_{1\text{-}40/42}$ peptides (Fig. 3). In the plaque area, numerous glial cells, mainly with the morphology of astrocytes, and less numerous, those 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 6F/3D negative indicating that they were the product of α-secretase. They were positive for the three other antibodies, Rabm38, Rabm40 and Rabm42, demonstrating that both astrocytes and microglia accumulate $A\beta_{17\text{-}40/42}$.

Intracellular Aβ distribution in neurons. The morphological diversity of Aβ deposits suggested that Aβ was present in different compartments of the lysosomal pathway and in lipofuscin in neuron type-specific amounts. The number and size of cathepsin D-positive lysosomes was from 2 to 3 times more than the number of Aβ-positive deposits. In cells strongly Aβ immunopositive, such as pyramidal neurons in the frontal cortex, approximately 50% of cellular Aβ (mAb4G8) was detected in cathepsin D-positive lysosomes (Fig. 5). Lamp 1 immunoreactivity was strong in all three examined regions, but only about one third of lysosomes in the frontal cortex, ~5% of lysosomes in Purkinje cells, and approximately 20% in the dentate nucleus revealed Aβ immunoreactivity.

In cortical pyramidal neurons, with moderate amounts of lipofuscin, co-localization of autofluorescent lipofuscin with ~20% of cytoplasmic Aβ was observed. In Purkinje cells, with only traces of lipofuscin, co-localization was found for less than 10% of Aβ, whereas in the dentate nucleus, with lipofuscin-rich neurons, co-localization was detected for ~40% of cytoplasmic Aβ (Fig. 6). The observed patterns suggest that the proportion of cytoplasmic Aβ in lipofuscin increases with the amount of cell lipofuscin.

DISCUSSION

The difference between the pattern of intracellular Aβ accumulation in the brain of control subjects and subjects diagnosed with idiopathic autism and autism/dup(15). The accumulation of intraneuronal Aβ is considered as a first step leading to amyloid plaque formation in AD (Gyure et al 2001, D'Andrea et al 2001, Mochizuki et al 2000, Gouras et al 2010). However, our examination of control brains during the life span showed that intraneuronal Aβ also occurs in normal controls, and revealed that almost all cytoplasmic Aβ peptides are the product of α- and γ-secretases ($A\beta_{17\text{-}40/42}$) (Wegiel et al 2007); whereas, the majority of amyloid in plaques is the product of β- and γ-secretases. This suggests that a brain region- and neuron type-specific patterns of intraneuronal $A\beta_{17\text{-}40/42}$ peptide accumulation in control brains, is a baseline for detection and evaluation of increases associated with autism, FXC, epilepsy, brain trauma or age-associated neurodegeneration, such as AD.

Trafficking and excessive accumulation of $A\beta_{17\text{-}24}$ in neurons. Aβ is generated in the endolysosomal pathway and in the endoplasmic reticulum/Golgi compartment (Glabe 2001, Greenfield et al 1999, Cook et al

1997, Hartmann et al 1997) and is also detected in multivesicular bodies (Takahashi et al 2002, Wilson et al 1999) and in mitochondria (Caspersen et al 2005, Bayer and Wirths 2010). The application of two markers of lysosomes, cathepsin D and Lamp1, 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 there exists a selective enhancement of target proteins hydrolysis by this aspartic acid protease (Sheikh et al 2010). The lysosome is the major acid hydroxylase-containing cell compartment engaged in processing of substrates delivered by (a) endocytosis, (b) autophagy (Gordon et al 1992), and (c) by the scavenging of proteins from the endoplasmic reticulum to lysosomes (Noda and Farquhar 1992). 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 another 20-30% of neuron $A\beta_{17-40/42}$ is present in lipofuscin, which is the final product of cytoplasmic proteolytic degradation of exo- end endogenous substrates. During the entire lifespan lipofuscin gradually accumulates in neurons (Brunk and Terman 2002a). The age of onset and dynamics of lipofuscin deposition are cell-type specific (Brody 1960, Bancher et al 1989). Our previous study revealed that neurons in the inferior olive, dentate nucleus, and the lateral geniculate body, start accumulating lipofuscin and $A\beta_{17-40/42}$ early in the life and that this accumulation progresses with age in region-specific rates (Wegiel et al 2007).

The pattern of both Aβ and lipofuscin accumulation can be dramatically modified in early childhood in subjects with autism and even more significantly in individuals with autism/dup(15). 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, 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 (2008). 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 (Jellinger et al (1988), an Autism Spectrum Disorder, as well as, in several psychiatric disorders including bipolar affective disorder (Yanik et al., 2004) and schizophrenia (Herken et al., 2001, Akyol et al., 2002).

Enhanced lipofuscin accumulation and enhanced $A\beta_{17-40/42}$ immunoreactivity in the majority of the examined brain structures in most of the autistic and dup(15) individuals 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 (Sohal and Brunk 1989; Brunk et al., 1992). The link between oxidative stress, cytoplasmic degradation and lipofuscin deposition is supported by the presence of oxidatively modified proteins and lipids in lipofuscin (Brunk and Terman 2002a,b; Terman and Brunk 2004). A significant increase of malondialdehyde levels (a marker of lipid peroxidation) in the plasma of autistic children (Chauhan et al 2004) and in the cerebral cortex and cerebellum (Chauhan and Chauhan 2010), 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, 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 (Pike et al 1995) 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 (Pigino et al 2009). Inhibition of FAT results from activation of endogenous casein kinase 2 (CK2). Altered regulation of FAT markedly reduces transport of synaptic proteins and mitochondria in the AD brain and in AD mouse models that accumulate oAβ (Pigino et al 2003). Dysregulation of FAT results in distal axonopathies with a reduced delivery of critical synaptic elements required for the integrity, maintenance, and function of synapses (Pigino et al 2009).

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 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 (Wei et al 2002). However, *in vitro* studies of the neuronal response to exogenous Aβ peptides do not replicate the neuronal exposure to endogenous $A\beta_{17\text{-}40/42}$ trafficking inside vesicles and vacuoles of lysosomal pathway.

$A\beta_{1\text{-}40/42}$ 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 $A\beta_{1\text{-}40/42}$ in plaques. It was hypothesized that $A\beta_{17\text{-}42}$ 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 (Gowing et al 1994, Pike et al 1995, Saido et al 1995). Gouras et al (2000) considered intracellular $A\beta_{42}$ accumulation as an early event leading to neuronal dysfunction. The $A\beta_{1\text{-}40/42}$ –

positive diffuse plaques in the brain of autistic subjects are different than the $A\beta_{17-40/42}$-positive cerebellar diffuse plaques detected in Down's syndrome (Gowing et al 1994, Lalowski et al 1996). Diffuse amorphous nonfibrillar Aβ deposits, called amorphous plaques (Rozemuller et al 1989), pre-plaques (Mann et al 1989) or pre-amyloid deposits (Tagliavini et al 1989) are considered to be of neuronal origin (Dickson 1997, Probst et al 1991, Wisniewski et al 1996, 1998) and are formed selectively in projection areas of distant affected neuronal populations (Wegiel and Wisniewski, 1999). 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 (Thal et al 1999). In astrocytes, intracellular Aβ appears in lysosomes and lipofuscin (Funato et al 1998, Yamaguchi et al 1998). 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 (Thal et al 1999).

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, produce a chronic traumatic encephalopathy with the associated deposition of Aβ, most commonly as diffuse plaques (DeKosky et al 2007, Gentleman et al 1997, McKee et al 2009). In acute traumatic brain injury, diffuse cortical Aβ deposits were detected in 30% to 38% of cases 2 hours after injury (Murakami et al 1998, Roberts et al 1994, Ikonomovic et al 2004).

In conclusion, this postmortem study of Aβ distribution in the brain of subjects with idiopathic autism and dup(15) autism suggests (a) a prevalence of anabolic α-secretase APP processing and $A\beta_{17-40/42}$ accumulation in neuronal lysosomes and lipofuscin in the majority of autistic children and adults, and (b) an activation of the amyloidogenic pathway of APP processing with β- and γ-secretases, and diffuse nonfibrillar plaques formation in some autistic subjects from 39 to 52 years of age.

Figures

Fig. 1. Mapping of $A\beta_{17-24}$ in the brain AN09402 reveals brain region and cell type specific pattern 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 and temporal cortex are 4G8-positive but the reaction intensity varies from minimal to very prominent. Very heavy immunoreactivity is observed in almost all neurons in the lateral geniculate body, thalamus, amygdala, Purkinje neurons and small cells in the molecular layer (most likely interneurons), in almost all neurons and astrocytes in the CA4, large and small neurons in the dentate nucleus. Some types of neurons (in the reticular nucleus in the thalamus and small neurons in the dentate nucleus) have two types of deposits: fine-granular and 2-3 μm in diameter dense 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 (α-secretase product). Immunoreactivity with mAb 4G8 is present in the brain of the control subject (14 years of age) but less neurons are positive and immunoreactivity in the frontal cortex, thalamus, cerebellum; and dentate nucleus is weaker than in the affected subject. Glial cells are immunonegative.

Fig. 2. Distribution of Aβ (mAb 4G8, green) in astrocyte cytoplasm (GFAP; red) in the frontal cerebral cortex of a 10 year old male diagnosed with dup(15), autism, early onset (8 month) intractable epilepsy and epilepsy related death (SUDEP) (AN06365). Aβ deposits are marked with arrows. Cell nuclei were stained with TO-PRO-3-iodide (blue). Occipital cortex of a 10 years of age subject with autism/dup15 characterized by the presence of 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 in perivascular space (e).

Fig. 3. Diffuse plaques in the frontal cortex of a 39 years old female (AN11931) diagnosed with dup(15), autism, intractable seizures (age of onset 9 years), 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 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 (α-secretase product).

Fig. 4. Diffuse plaques in the frontal cortex of a 51 year old subject (AN17254) diagnosed with idiopathic autism, who had only one grand mal seizure and died because of cardiac arrest, are immunopositive when stained with all five antibodies (6E10, 4G8, Rabm38, 40, and 42) but granular material in the cytoplasm of glial cells is immunopositive for all antibodies used except 6E10 (α-secretase product).

Fig. 5. Co-localization of Aβ (mAb 4G8; green) with markers of lysosomal pathway: cathepsin D (red) in the frontal cortex (FC), and Lamp1 (red) in the frontal cortex (FC), cerebellum (Crb/PC), and dentate nucleus (DN) of a 10 year old male diagnosed with autism/dup(15) (AN06365) illustrates the neuron-type specific patterns of Aβ distribution in lysosomal pathway. The number and size of cathepsin D-positive lysosomes is approximately 2-3 times more than the Aβ-positive granules. Approximately 50% of Aβ is colocalized with cathepsin D-positive lysosomes. In the frontal cortex (FC), about one third of Lamp1-positive lysosomes are Aβ positive and the majority of Aβ is in present in the lysosomes. In Purkinje cells (PC), the lysosomes are numerous but only a few (~5%) are Aβ positive. The majority of Purkinje cells Aβ is not associated with lysosomes. In the dentate nucleus, both Aβ deposits and lysosomes are numerous, but only ~20% of Aβ is co-localized with lysosomes.

Fig. 6. Co-localization of Aβ (4G8, green) with autofluroescent (red) lipofuscin in the frontal cortex (FC), cerebellar cortex (CrbC), and dentate nucleus (DN) of a 10 years old subject diagnosed with autism/dup(15) (AN06365), demonstrates that the portion of cellular Aβ is stored in the end stage of the proteolytical pathway. The percentage of Aβ co-localized with lipofuscin is ~20% in neurons in the frontal cortex, ~5% in the Purkinje cells, but in the dentate nucleus ~50% of Aβ is detected in the lipofuscin. The arrowhead marks the Aβ located in the autofluorescent deposits of lipofuscin.

Table 1. Material examined, cause of death, epilepsy.

SUDEP, sudden unexpected and unexplained death of subject with known epilepsy.

IE, intractable epilepsy. E, epilepsy.

Group	Brain bank number	Sex	Age (y)	Cause of death	Epilepsy. Age of onset
dup(15)	AN14762	M	9	SUDEP	IE/10m
dup(15)	AN06365	M	10	SUDEP	IE/8m
dup(15)	AN09402	M	11	SUDEP	IE/10m
dup(15)	AN07740	F	15	SUDEP (suspected)	E/11y
dup(15)	AN09470	F	15	Pneumonia	-
dup(15)	AN03935	M	20	Cardiopulmonary arrest	-
dup(15)	AN05983	M	24	Pneumonia	IE/7y
dup(15)	AN14829	F	26	SUDEP (suspected)	E/16y
dup(15)	AN11931	F	39	SUDEP	IE/9y
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/8y
Autism	AN01293	M	9	Cardiac arrest	-
Autism	CAL105	M	11	Asphyxia (drowning)	E
Autism	IBR93-01	M	23	Seizure related	E/23y
Autism	AN08166	M	28	Seizure-related	E
Autism	NP06-54	M	32	Brain tumor	-
Autism	AN17254	M	51	Cardiac arrest	1 grand mal
Autism	BB1376	M	52	Cardiac arrest	-
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	-

Table 2. Antibodies used for immunocytochemistry (Ic) and immunofluorescence (confocal microscopy –Conf). Host: Rabbit monoclonal (R-m) or polyclonal (R-p); Mouse monoclonal (M-m)

Name	Epitope or target	Dilution	Host	Source
6E10	4-13 aa Aβ	1:10,000	M-m	Signet Laboratories (antibody developed at IBR) (Kim et al 1990, Miller et al 2003)
6F/3D	8-17 aa Aβ	1:50	M-m	Novocastra
4G8	17-24 aa Aβ	1:8,000	M-m	IBR (Kim et al 1988)
Rabm38	-38 aa Aβ	100 ng/mL	R-m	(Miller and Mehta)
Rabm40	-40 aa Aβ	100 ng/mL	R-m	Miller et al 2010
Rabm42	-42 aa Aβ	100 ng/mL	R-m	Miller et al 2010
Cathepsin D	Lysosomes	1:100	R-p	Calbiochem
LAMP 1	Lysosomes/ Autophagosomes	1:400	R-p	Abgent
GFAP	Astrocytes	1:400	R-p	Sigma

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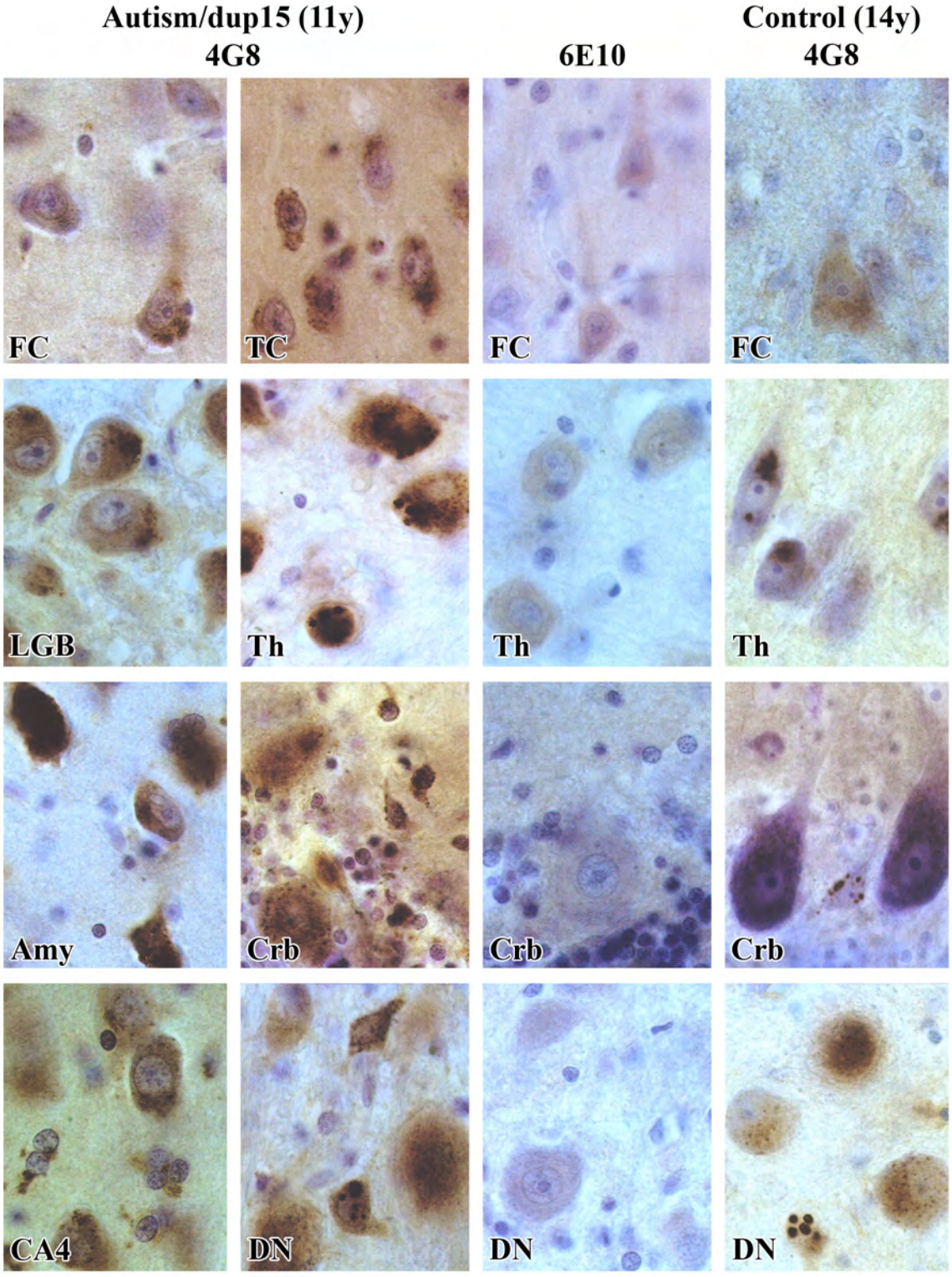
Autism/dup15 (11y)
4G8
6E10
Control (14y)
4G8
FC
TC
FC
FC
LGB
Th
Th
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Amy
Crb
Crb
Crb
CA4
DN
DN
DN

Fig. 1

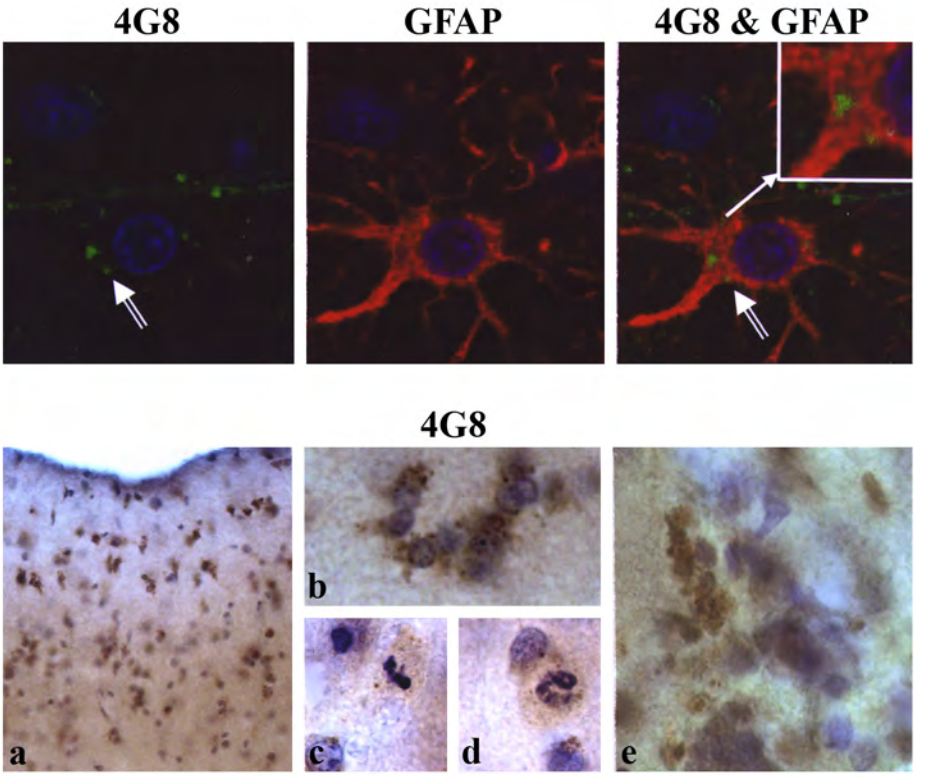

Fig. 2

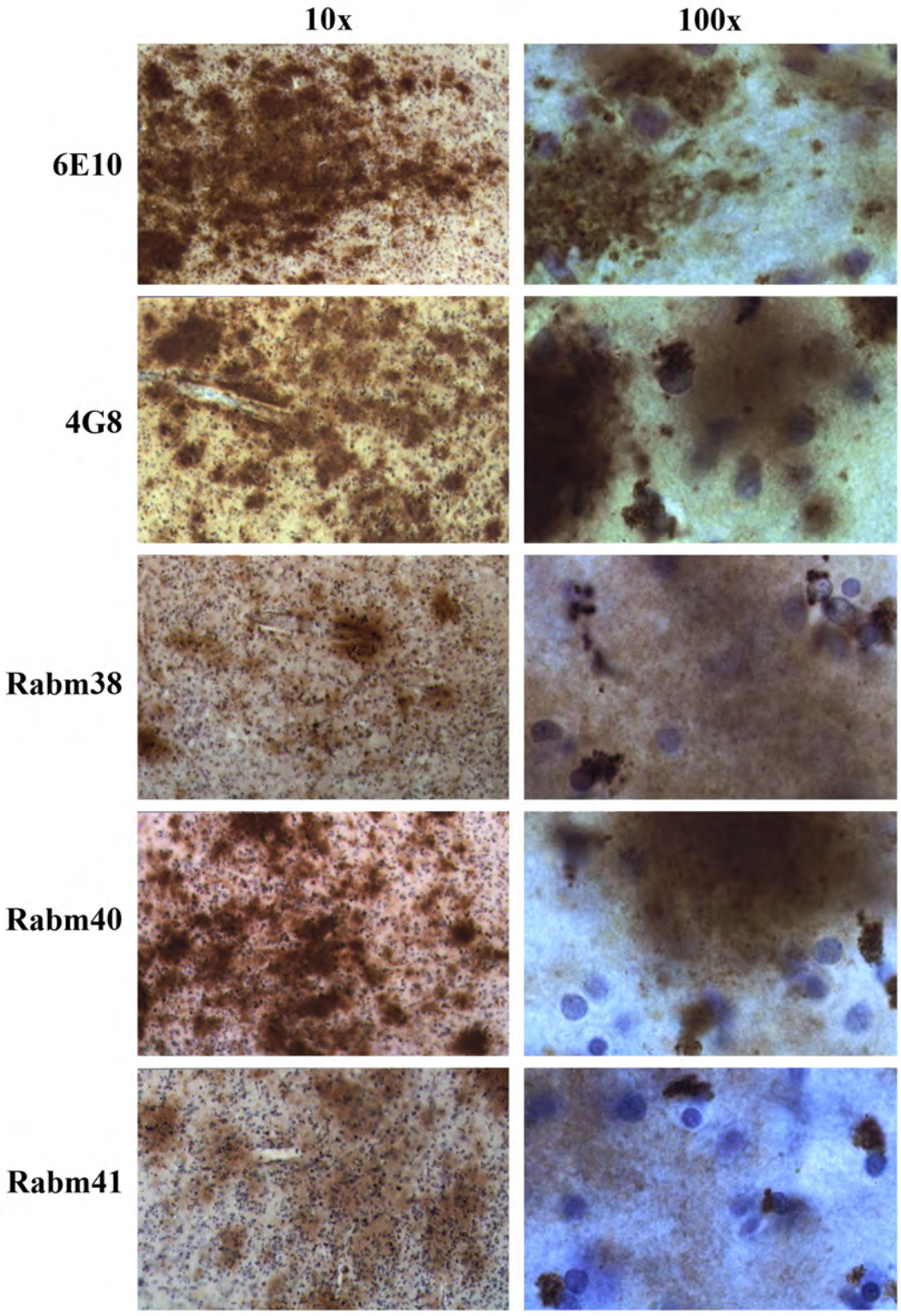

Fig. 3

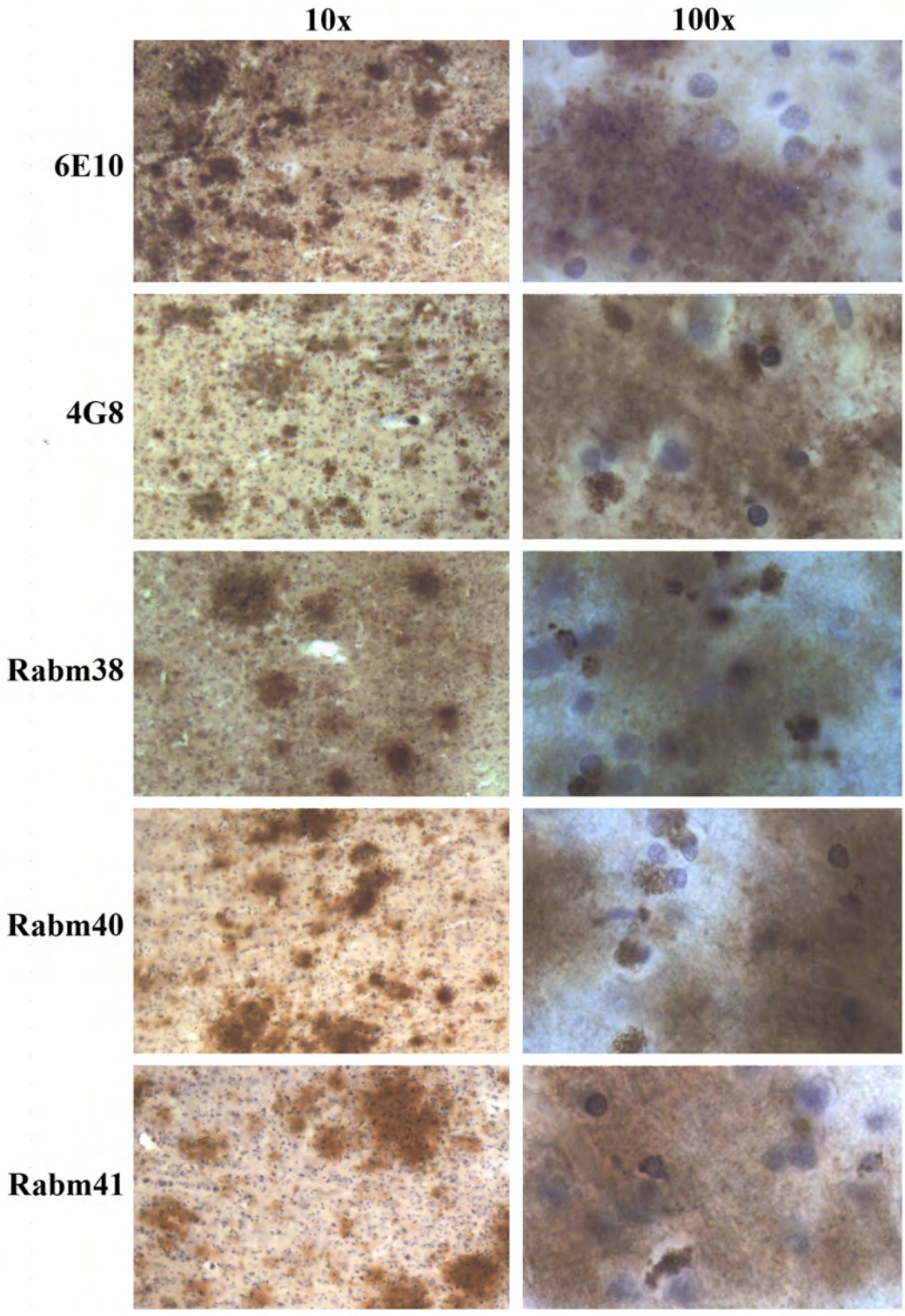

Fig. 4

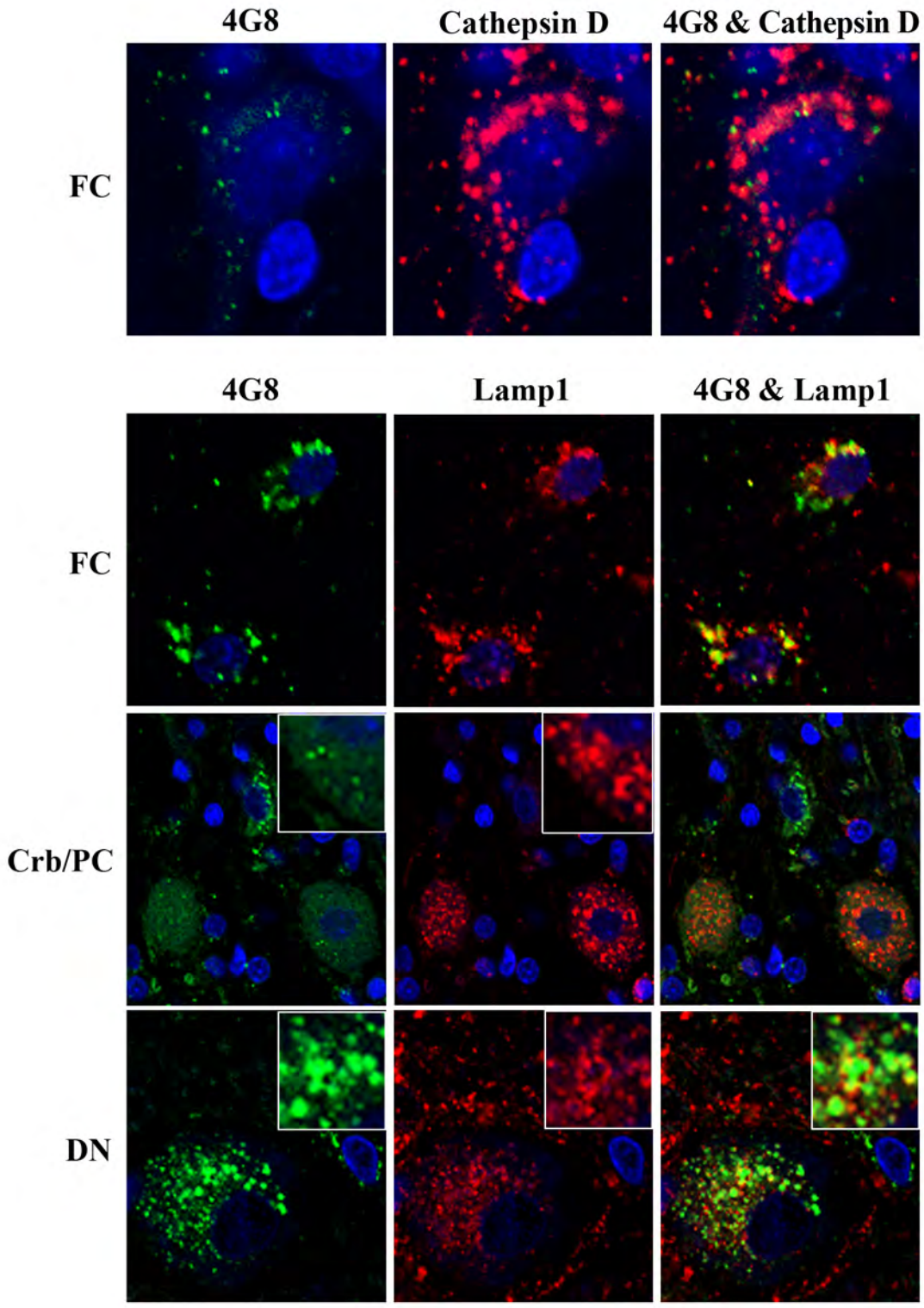
4G8
Cathepsin D
4G8 & Cathepsin D
FC
4G8
Lamp1
4G8 & Lamp1
FC
Crb/PC
DN

Fig. 5

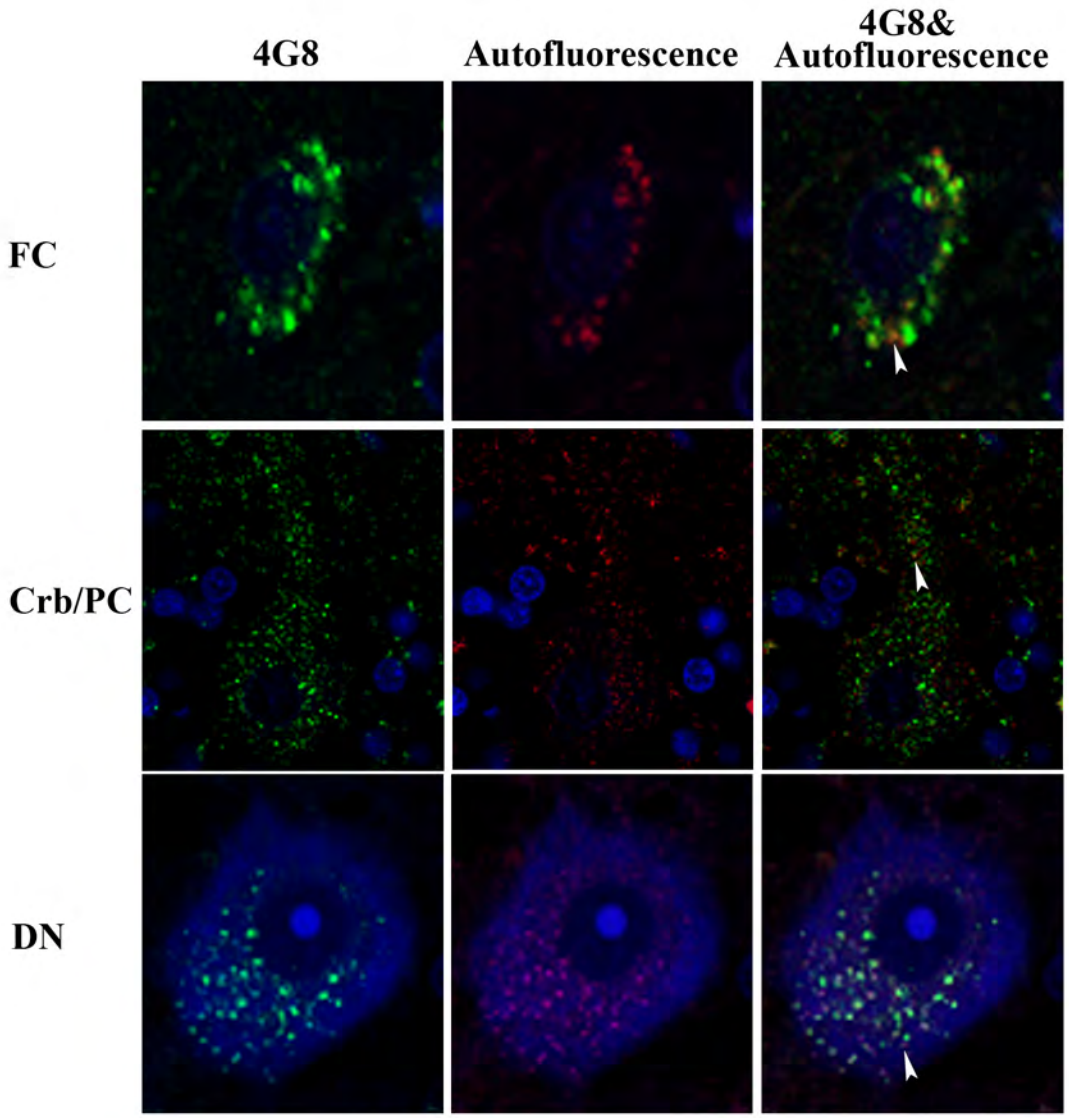

Fig 6