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

Subproject 1: The neuropathological markers of abnormal brain development and aging in autism.

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SUBPROJECT 1

The neuropathological markers of abnormal brain development and aging in autism

Subproject 1 P.I.: Thomas Wisniewski, M.D.

INTRODUCTION

The study of neuropathological, morphometric and biochemical markers of autism was designed to detect correlations between pathology and clinical manifestations of autism, and to identify developmental alterations that might be targets for treatment.

The project was executed consistent with the original Program Project and Project 1 aims and timetable.

Material: We examined brains of 72 subjects including: 32 brains of autistic people, 12 brains of individuals with autism associated with chromosome 15 duplication (dup15) and 28 brains of control subjects, exceeding the original plan by approximately 30%.

Aims: Project 1 plays a dual role in the Program Project and its function is reflected in the technical and research aims:
1. To preserve tissue from 72 brains, according to a standardized protocol for neuropathological studies (Project 1) and for morphometric studies (Project 2).
2. To implement clinical inclusion and neuropathological exclusion criteria to reduce the risk of distortion of morphometric studies by comorbidity, postmortem tissue changes, and pathology associated with mechanisms leading to death. Cases with signs of comorbidity, premortem and postmortem changes affecting brain structure were excluded from the morphometric studies.
3. To determine the type, topography and severity of developmental changes including defects of neurogenesis, migration and dysplastic changes, and to estimate their contribution to epilepsy and epilepsy related mortality.
4. To establish protocols of tissue preservation for neuropathological and morphometric studies of two regulatory systems: serotonergic system and hypothalamic system with known contribution to behavioral changes in autism and with a crucial role in trophic control of neuronal growth in the entire brain.
**Tissue preservation.** Tissue preservation included: brain hemisphere fixation, dehydration, embedding, cutting, staining/immunostaining. Two standardized protocols were applied: celloidin and polyethylene glycol (PEG) embedding protocols. The celloidin protocol provides 200-µm-thick sections mainly for brain neuropathology and morphometry. The PEG protocol provides 50-µm-thick sections for neuropathology and immunocytochemistry-based morphometry. Application of both protocols results in preservation of the entire brain hemisphere for a unique power (a) extended protocol of neuropathological evaluation detecting each abnormality more than 1 mm in diameter and (b) complex morphometric study of 36 brain anatomic structures and their cytoarchitectonic subdivisions selected to monitor potential link between structural developmental defects and social and communication deficits, ritualistic behaviors, intellectual deficits, and seizures.

**Data acquisition, storage and processing.** Tissue processing, distribution and analysis were monitored by a computerized system of brain tissue samples, sections and histological slides trafficking and storage. This Neuropathological Database was linked to the Project 2 Morphometric Database.

**BODY**

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

   Approximately 5%–10% of individuals with an ASD have an identifiable genetic etiology corresponding to a known single gene disorder, such as fragile X syndrome, or chromosomal rearrangements, including maternal duplication of 15q11-q13. The symptoms in people with idic(15) markers are correlated with the extent of duplication of the Prader Willi syndrome/Angelman syndrome critical region (PWS/ASCR) (15q11-q13) (Cheng et al 1994, Robinson et al 1993). Larger supernumerary idic(15) chromosomes, are associated with a cluster of clinical features that include autistic behavior, intellectual deficit (ID), seizures, hypotonia, hyperactivity, and irritability (Wisniewski et al 1979). Duplications of chromosome 15q11-q13 account for approximately 0.5%–3% of ASD and may be the most prevalent cytogenetic aberration associated with autism in most studies (Cook et al 1997, Schroer et al 1998, Gillberg et al 1991, Ghaziuddin et al 1993, Baker et al 1994, Bunday et al 1994).

   We hypothesized that neuropathology of autism with dup(15) differs from that of idiopathic autism and provides an explanation for the high prevalence of seizures and associated sudden death in dup(15) cohort. The purpose of this study was to detect the difference between the patterns of developmental abnormalities in the brains in autism of unknown etiology (idiopathic autism) and individuals with duplications of chromosome 15q11.2-q13 [dup(15)] and autism, and to identify alterations that may contribute to seizures and sudden death in dup(15) syndrome. Brains of the 9 subjects with dup(15), 10 with idiopathic autism and 7 control subjects were examined. In dup(15) cohort seven subjects (78%) were diagnosed with autism, 7 had seizures (78%), and in 6 cases (67%) sudden unexplained death was reported. Subjects diagnosed with dup15 autism were microcephalic, with the mean brain weight 300g less (1,177 g) than in idiopathic autism (1,477 g; p<0.001). Heterotopias in the alveus, CA4 and dentate
gyrus, and dentate gyrus dysplasia were detected in 89% of subjects with dup(15) autism but in only 10% of subjects with idiopathic autism (p<0.001). However cerebral cortex dysplasia was detected in 50% of subjects with idiopathic autism and in none of dup(15) autism cases (p<0.04). Different spectrum and higher prevalence of developmental neuropathological changes than those seen in idiopathic autism may contribute to high risk of early onset of seizures and sudden death in the dup(15) cohort.

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


Postmortem studies of brains of individuals with idiopathic autism and duplications 15q11.2-q13 autism identify a cluster of neuropathological features differentiating these cohorts. They show a need for both sub-classification of autism according to etiology, clinical presentation, and neuropathology, and a commonality of clinical and neuropathological traits justifying autism diagnosis. The features differentiating these cohorts include: (a) maternal origin dup(15), (b) autism in 78% of subjects, (c) more severe clinical phenotypes, with intellectual deficit (100%), early-onset of severe or intractable seizures in 78% of subjects, and increased to 67% prevalence of sudden unexplained death, (d) high prevalence of microcephaly, with mean brain weight 300g less than in idiopathic autism, (e) several-fold increase in the number of developmental abnormalities, including defects of migration and dysplastic changes, especially numerous in the hippocampal formation, and (f) significant increase of the intraneuronal amyloid load, reflecting enhanced amyloid-β precursor protein processing with α-secretase.

2. Developmental alterations of raphe nuclei in autistic subjects 5-15 years of age –

Methods and technical limitations.
Wegiel Jarek, CUNY, Ph.D. Dissertation 2013, pp 1-135

The role of the serotonergic system in autism is supported by more than 500 reports. They reveal a link between serotonergic system alterations and social deficits, repetitive behavior, hyperactivity, anxiety and obsessive compulsive behavior observed in autism:

2. The association between hyperserotonemia and increased risk of recurrence of autism within families (Cook et al. 1990, Piven et al. 1991, Cross et al. 2008).
3. The correlation between blood serotonin level and severity of clinical symptoms (Hérault et al. 1996).
4. The correlation between low level of serotonin precursor (tryptophan) and severity of stereotyped behaviors in autistic subjects (McDougle et al. 1996).
6. Link between brain serotonin deficit and severity of social deficits (Chugani et al. 1999)
7. Link between reduced uptake of tryptophan and severe language deficits (Chandana et al. 2005).

Abnormalities of serotonergic fibers in many target cortical and subcortical structures known to be involved in autism phenotype (Azmitia et al. 2011a).

Azmitia (2011b) review reveals links between serotonergic system alterations and epilepsy, immune dysregulation, gastrointestinal and sleep disorders, as well as anxiety, aggression, obsessive compulsive behavior and mood disorders observed in autism. However, in spite of evidence of altered development of brain serotonergic system and contribution of these alterations to the autism phenotype, the raphé nuclei, which are the source of brain serotonin, have not been examined.

The aim of this stereological and quantitative immunofluorescence-based study of raphé nuclei in autistic subjects 5 to 15 years of age and age matched control subjects was to (a) establish methods of preparation, staining, and analysis of fixed human brainstem samples obtained from brain banks, and (b) characterize the pattern of developmental abnormalities which may contribute to the autistic phenotype.

Routine neuropathological brainstem dissection results in partial or complete loss of raphé nuclei integrity. From 9 autistic and 6 control subjects only four pairs 5 to 15 years of age were qualified for the study of raphé nuclei. Formalin-fixed brainstem was dehydrated and embedded in polyethylene glycol and cut into serial 50-µm-thick sections. They were stained to estimate cell volume, and immunostained and examined by fluorescence microscopy to estimate the amount of tryptophan hydroxylase (TPH) which is a measure of serotonin synthesis level.

3-D reconstruction demonstrated topography and size of raphé nuclei and explained why preservation of raphé nuclei located in the midline required modification of brainstem sampling. Nucleator applied to TPH (+) sections revealed 24% smaller neuronal soma volume in the dorsal raphé nuclei of autistic subjects than in control group. Application of immunofluorescence and ImageJ software (NIH) revealed significant increase in tryptophan hydroxylase (TPH) immunofluorescence in spite of smaller size of raphé neurons.

These data indicate developmental impairment of neuron growth comparable to that observed in cortex and in subcortical structures. Enhanced TPH immunofluorescence in raphé neurons was consistent with enhanced immunoreactivity in serotonergic fibers in several brain regions of autistic subjects (Azmitia et al. 2011ab). Pathology detected in raphé neurons suggests that target brain areas were exposed to altered levels of serotonin, which may modify function of cerebral cortex and subcortical structures and contribute to the autistic phenotype.
**KEY RESEARCH ACCOMPLISHMENTS**

Project 1 has a significant contribution to three major research strategies of the Program Project, 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

Major accomplishments:

1. Because of DOD grant and Autism Speaks and Autism Tissue Program support of tissue acquisition, we were able to preserve the historically largest collection of unique quality brain tissue samples (72 brain hemispheres) including:
   - 32 brain hemispheres of people with idiopathic autism,
   - 12 brain hemispheres of people with dup15 autism,
   - 28 control brain hemispheres.
2. Brain hemispheres cut into serial sections provided material for several research strategies. Therefore, we were able to expand the spectrum of research targets and hypotheses tested in this Program Project.
3. The neuropathological component (Project #1) provided neuropathological reports and exclusion criteria reducing risk of distortion of research results by comorbidity, pre-, peri- and postmortem changes.
4. The study determined the contribution of qualitative developmental abnormalities to the autistic phenotype in autism with unknown etiology and autism caused by maternal origin dup(15) (Wegiel et al 2010, 2012).
5. We identified both (a) differences between the pattern of developmental abnormalities in autism associated with duplications 15q11.2-q13 and autism of unknown origin, and (b) the core neuropathology present in autism regardless of autism etiology.
6. This project results in detection of the global pattern of focal abnormalities distribution and the link between these alterations and early onset of epilepsy, functional regression and an increased risk of Sudden Unexpected Death in Epilepsy (SUDEP).

**REPORTABLE OUTCOMES**

**Publications**


Meetings

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


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


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

11. Hypothalamic neurons developmental delay in autistic subjects. Ma SY, Kuchna I,
CONCLUSIONS

Focal dysplasia and heterotopias are a major cause of increased risk of epilepsy and sudden unexpected death in autism, especially in autism associated with dup15

1. Idiopathic autism is associated with focal developmental abnormalities in 92% of cases including:
   (a) Multifocal cerebral neocortical dysplasia observed in 8% of subjects;
   (b) Multifocal archicortex dysplasia in 15%;
   (c) Cerebellar dysplasia in 62%;
   (d) Subcortical, periventricular, hippocampal and cerebellar heterotopias reflect abnormal neuronal igration in 31%;
   (e) Thickenning of the subependymal cell layer and subependymal nodular dysplasia and an indicative of active neurogenesis in autistic children.

2. Dup(15) autism is associated with enhanced developmental alterations:
(a) Severe microcephaly, with brain weight reduced by 300 g, is one of the most significant signs of global encephalopathy increasing the risk of epilepsy.

(b) 2.8 times more frequent developmental alterations, especially common in the hippocampal formation of autistic subjects with dup15, and presence of up to 11 different types of developmental alterations in the brain of a single subject diagnosed with dup(15) autism are a major contributor to early onset of epilepsy and high risk of SUDEP.

(c) Reduced volume of neurons in a majority of subcortical structures and some cortical regions in the brain of autistic children 4–8 years of age appear to reflect brain immaturity in early childhood contributing to autism and intellectual deficits.

(d) Combination of all of these developmental defects increases risk of death at a very early age (~10 years) in autism associated with dup15.

Neuropathology of serotonergic system in autism.

1. This first study of raphé nuclei of 5 to 15 year old autistic subjects revealed:
   a) Significant interindividual differences of the pattern of morphological changes, most likely reflecting contribution of genetic and pre- and postnatal alterations to postmortem detected changes.
   b) Increase in TPH immunoreactivity per cell body that appears to reflect increased level of serotonin in the interfascicular nucleus.
   c) Reduced volume of neuron soma in DRN consistent with numerous reports published in the past two decades demonstrating small neuron size in cerebral cortex, subcortical structures, and cerebellum in autistic subjects.

2. The study recognized several limitations of research on raphé nuclei of autistic subjects including:
   a) Low number of postmortem brain donations of autistic subjects;
   b) Limited number of pediatric control donations;
   c) Conflict between routine methods of brainstem dissection and the need for preservation of anatomical integrity of brainstem serotonergic system for postmortem studies.

3. Standardization of brainstem preservation, immunostaining and methods of quantitative analysis, especially immunofluorescence-based measurements of key proteins reflecting raphé structure and function, will contribute to detection and characterization of serotonergic system alterations in autism.

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Azmitia EC, Singh JS, Whitaker-Azmitia JM. Increased serotonin axons (immunoreactive to 5-HT transporter) in postmortem brains from young autism donors. Neuropharmacology 2011b, 60, 1347-1354


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APPENDICES


