

Neuron Number and Size in Prefrontal Cortex of Children With Autism

Eric Courchesne, PhD

Peter R. Mouton, PhD

Michael E. Calhoun, PhD

Katerina Semendeferi, PhD

Clelia Ahrens-Barbeau, BS

Melodie J. Hallet, MS

Cynthia Carter Barnes, PhD

Karen Pierce, PhD

CLINICAL SIGNS OF AUTISM ARE often preceded by or emerge concurrently with a period of abnormal brain and head overgrowth.¹⁻¹² This early neurobiological signal of abnormal development has been reported to begin at 9 to 18 months of age.^{2,9-11}

Overgrowth¹³ and neural dysfunction^{14,15} are evident at young ages in multiple brain regions, including the prefrontal cortex (PFC),^{3,6,7,11,12} that are involved in higher-order social, emotional, communication, and cognitive development. Therefore, knowledge of the neural basis of overgrowth could point to early causal mechanisms in autism and elucidate the neural functional defects that engender autistic symptoms. In the first magnetic resonance imaging (MRI) report of early brain overgrowth in autism a decade ago, it was theorized that excess numbers of neurons could be an underlying cause, perhaps due to prenatal dysregulation of proliferation, apoptosis, or both.¹

However, the neural basis of early overgrowth remains unknown and can

For editorial comment see p 2031.

Author Audio Interview available at www.jama.com.

Context Autism often involves early brain overgrowth, including the prefrontal cortex (PFC). Although prefrontal abnormality has been theorized to underlie some autistic symptoms, the cellular defects that cause abnormal overgrowth remain unknown.

Objective To investigate whether early brain overgrowth in children with autism involves excess neuron numbers in the PFC.

Design, Setting, and Cases Postmortem prefrontal tissue from 7 autistic and 6 control male children aged 2 to 16 years was examined by expert anatomists who were blinded to diagnostic status. Number and size of neurons were quantified using stereological methods within the dorsolateral (DL-PFC) and mesial (M-PFC) subdivisions of the PFC. Cases were from the eastern and southeastern United States and died between 2000 and 2006.

Main Outcome Measures Mean neuron number and size in the DL-PFC and M-PFC were compared between autistic and control postmortem cases. Correlations of neuron number with deviation in brain weight from normative values for age were also performed.

Results Children with autism had 67% more neurons in the PFC (mean, 1.94 billion; 95% CI, 1.57-2.31) compared with control children (1.16 billion; 95% CI, 0.90-1.42; $P=.002$), including 79% more in DL-PFC (1.57 billion; 95% CI, 1.20-1.94 in autism cases vs 0.88 billion; 95% CI, 0.66-1.10 in controls; $P=.003$) and 29% more in M-PFC (0.36 billion; 95% CI, 0.33-0.40 in autism cases vs 0.28 billion; 95% CI, 0.23-0.34 in controls; $P=.009$). Brain weight in the autistic cases differed from normative mean weight for age by a mean of 17.6% (95% CI, 10.2%-25.0%; $P=.001$), while brains in controls differed by a mean of 0.2% (95% CI, -8.7% to 9.1%; $P=.96$). Plots of counts by weight showed autistic children had both greater total prefrontal neuron counts and brain weight for age than control children.

Conclusion In this small preliminary study, brain overgrowth in males with autism involved an abnormal excess number of neurons in the PFC.

JAMA, 2011;306(18):2001-2010

www.jama.com

only be known from direct quantitative studies of the young postmortem autistic brain. In one study, 4 postmortem cases of 4- to 11-year-olds with autism had approximately 53% more Von Economo neurons in the frontoinsular cortex than 3 controls.¹⁶ In another study, the brain of a 3-year-old with autism had 58% more Von Economo neurons than that of a 2-year-old control.¹⁷ Since the total number of Von Economo neurons in the brain is small, an excess of these specific cell types cannot account for early brain overgrowth.

We stereologically quantified total neuron counts in 2 of the 3 major divisions of the PFC, which comprises

Author Affiliations: Department of Neuroscience, NIH-UCSD Autism Center of Excellence, School of Medicine, University of California San Diego, La Jolla (Drs Courchesne, Barnes, and Pierce and Mss Ahrens-Barbeau and Hallet) and Department of Anthropology (Dr Semendeferi), University of California San Diego, La Jolla; Department of Pathology & Cell Biology, University of South Florida School of Medicine, Alzheimer's Institute and Research Center, Tampa (Dr Mouton); and Sing Systems Inc, Silver Spring, Maryland (Dr Calhoun).

Corresponding Author: Eric Courchesne, PhD, Department of Neuroscience, NIH-UCSD Autism Center of Excellence, School of Medicine, University of California San Diego, La Jolla, CA 92093 (ecourchesne@ucsd.edu).

Table 1. Diagnostic Characteristics of Study Cases With Autism^a

Case	Age, y	ADI-Social ^b	ADI-Communication ^c	ADI-Restrictive and Repetitive ^d	Intellectual Disability
1	2	14	9	6	No ^e
2	3	20	8	3	No ^f
3	3	22	14	8	Yes ^e
4	4	14	10	3	Yes ^f
5	7	29	14	3	Yes ^f
6	8	19	7	4	No ^f
7	16	29	14	7	Yes ^e

Abbreviation: ADI-R, Autism Diagnostic Interview-Revised.

^aAll cases were male. All cases met or exceeded cutoffs for a diagnostic classification of autism using the ADI-R instrument.^bQualitative abnormalities in reciprocal social interaction (cutoff, 10; maximum score, 30).^cQualitative abnormalities in communication (cutoff, 7; maximum nonverbal communication score, 14).^dRestricted, repetitive, and stereotyped patterns of behavior (cutoff, 3; maximum score, 12).^eIntellectual disability status was determined using available standardized IQ scores (which have an intellectual threshold for intellectual disability on a standardized measure of intelligence of 70 or lower; a mean score of 100).^fIn the absence of a standardized IQ score, determination of intellectual disability was made based on review of specific questions and responses about verbal communication, expression abilities, and adaptive behavior skills on the ADI-R Narrative.

about one-third of all of the cortex, in autistic children compared with controls. Additionally, to determine whether excess prefrontal neuron counts in autism co-occur with abnormally enlarged brains, we compared brain weight in the autistic children with age-based normative weights and with brain weight in the controls.

METHODS

Brains were obtained from the National Institute of Child Health and Human Development (NICHD), University of Maryland Brain and Tissue Bank, the Autism Tissue Program at the Harvard Brain Tissue Resource Center, and the New York State Institute for Basic Research in Developmental Disabilities. Young postmortem cases are scarce and especially so with regard to tissue suitable for modern stereological study of the entire dorsolateral (DL-PFC) and mesial (M-PFC) subdivisions of the PFC. Such unbiased cell counting procedures are necessary to ensure valid cell counts, which cannot be obtained via density estimates from small blocks of cortical tissue.^{18,19} Brains were obtained from 7 autistic and 6 control male children aged 2 to 16 years, representing all young control male cases available at the time of the study and nearly all known young autism cases that had had the whole PFC uniformly sectioned. Cases were not se-

lected for any reason such as autopsy brain weight, postmortem interval (PMI), or cause of death, except that the PFC met requirements for performing valid stereological procedures.

Cases were from the eastern and southeastern United States and dates of death ranged from 2000 to 2006. Perinatal and postnatal medical conditions were obtained by the tissue banks from next of kin. Cause of death, PMI, and neuropathology were obtained from coroner's reports. Race of each postmortem case was determined by the tissue banks from information gathered from next of kin, and race and ethnicity categories were based on National Institutes of Health (NIH) requirements. Research procedures were approved by the institutional review board of the University of California, San Diego. Informed consent or waiver of consent was not required because all cases were deceased and deidentified and anonymized by the tissue banks.

All autism diagnostic classifications (TABLE 1) were based on the results of postmortem administration of the Autism Diagnostic Interview-Revised (ADI-R) to a parent or legal guardian of the deceased by a psychologist, which is the standard method for autism postmortem research. The ADI-R is a standardized parent interview used for determining developmental history and behavior for the pur-

poses of diagnosing autism. Questions are designed to elicit relevant information through queries closely associated with diagnostic criteria set forth in the *Diagnostic and Statistical Manual of Mental Disorders* (Fourth Edition; *DSM-IV*). The administration, scoring, and diagnostic determination are the same as when it is administered to the parent or legal guardian of a living individual. The psychologists who determined the intellectual ability level of each autistic child was blinded to knowledge of the neuropathology and neuron counts. Nonintellectual disability was defined as IQ of 71 or greater on standardized IQ tests or evidence from the ADI-R narrative of understanding of most words and sentences, communicative use of words and language, and some initiation of appropriate activities such as looking at books, using computers, showing some interest in mother, or playing games. Intellectual disability was defined as IQ equal to 70 or lower or little to no understanding or use of words, lack of appropriate activities, presence of self-injurious behavior, and/or nonresponsiveness to others.

All control cases were from the NICHD Brain and Tissue Bank. This tissue bank determined control cases to be free of mental illness, intellectual disability, and neurological disorder, including autism, based on information gathered in a detailed questionnaire at the time of death from next of kin. Cases with a history of chemotherapy or radiation treatment or being resuscitated following ischemic hypoxia were excluded as controls by the tissue banks.

Age-Based Normative Mean Brain Weights

Brain weight in normal male individuals increases with age.⁵ To test whether brain weight in the autistic children in the present study exceeded normal mean weight for age, the brain weight of each case was compared with the normative mean weight for age and expressed as a percent difference from that normative mean. To determine whether control children had brain weights expected for typical individuals, each weight was likewise compared to the normative mean

weight for age and expressed as a percent difference. The resulting age-based percent differences in brain weight within and between study groups were then compared. The age-based normative brain weights for males (eTable 2, available at <http://www.jama.com>) are based on approximately 11 000 cases reported in 10 normative brain weight studies.⁵

Anatomic Delineations of Prefrontal Subdivisions

We analyzed the DL-PFC and the M-PFC, 2 of the 3 major prefrontal subdivisions (FIGURE 1; eAppendix); orbital PFC was not measured. Anatomists

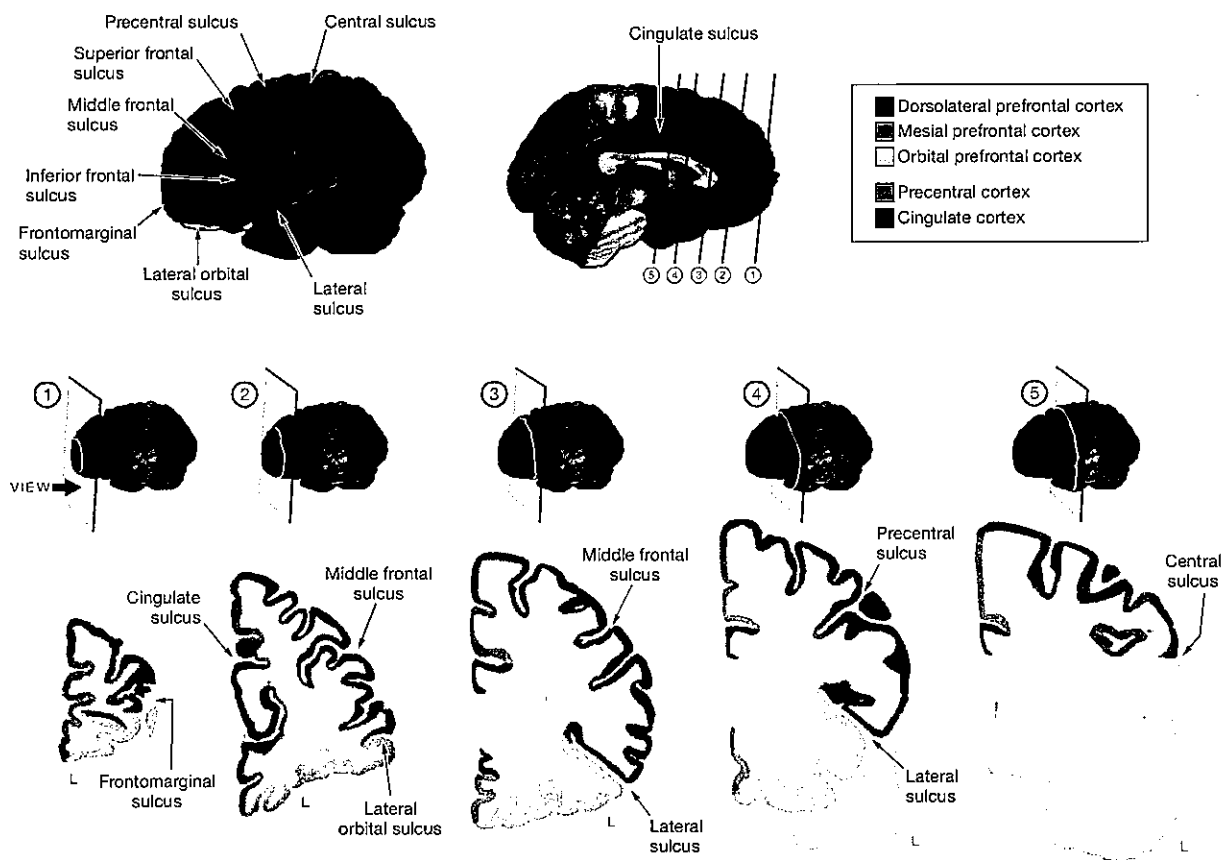
identified all anatomical boundaries of DL-PFC and M-PFC, blinded to diagnostic membership, age of case, and the purpose, literature, and theories associated with this study to ensure unbiased anatomic decisions. Anatomic delineation of the DL-PFC and M-PFC regions throughout their rostrocaudal extent was based on previous definitions^{6,20} and on overall gross anatomy, including tracking of sulci. Reliable boundaries were further refined based on cytoarchitectonic criteria,^{21,22} including layer 4 granularity and the presence of Betz cells; the overall cortical width and layer 6/white-matter transition; and density and clarity of cortical

columns were also used in some cases (eAppendix).

Stereology Procedures

Brains were serially sectioned and prepared for stereologic analysis (eAppendix; eTable 1). Quantifications of neuron number and mean cell volume within the DL-PFC and M-PFC were carried out blind to diagnostic membership, age of case, and the purpose, literature, and theories behind this study to ensure unbiased anatomic measurement. The sum of these 2 subdivisions gives the combined prefrontal neuron counts. Microglia and satellite oligodendrocytes (small nonmyelinated

Figure 1. Schematic of Dorsolateral Prefrontal Cortex and Mesial Prefrontal Cortex



Schematic of 2 prefrontal subregions, dorsolateral prefrontal cortex (green) and mesial prefrontal cortex (pink). Within each region, neuron counts, neuron size, and glia counts were performed using blinded stereological methods.

ing oligodendrocytes found in close association with large cortical neurons) were selected as nonneuronal contrast cell types and counted. Because in normal brain development glial cell proliferation continues well beyond the cessation of neural proliferation,²³ glia counts provided information on whether excess numbers of nonneuronal as well as neuronal cell types might occur in autism. The limitation of this procedure is that the Nissl stained sections do not enable microglia and satellite oligodendrocytes to be separately counted; glia counts, therefore, represent both glia cell types.

Counting

An optical fractionator method,²⁴ which is independent of volumetric shrinkage of tissue, was used to estimate the total number of neurons in each of the entire DL-PFC and M-PFC volumes using thin focal plane optical scanning (40-100 \times oil immersion), according to methods detailed previously.^{25,26} Briefly, 100 or more locations were sampled in the x- and y-axes on 8 to 12 sections per reference space. Neurons were distinguished from glia based on prominent nucleolus, clear nuclear membrane, and high cytoplasm-to-nucleus ratio. Neurons were counted according to Gundersen unbiased counting rules, with optical disector height and guard zone of 10 μ m and more than 8 μ m, respectively. Total neuron number was calculated with the optical fractionator method and sampling continued to a coefficient of error of 10% or less ($CE \leq 0.10$). Neuronal density for each reference space was calculated as the total neuron number divided by the product of total disector number and the disector volume.

Volume Measurement

After neurons were sampled and counted using the unbiased disector method,²⁷ the mean cell volume (MCV) of neurons was estimated using the rotator method.²⁸ The estimate of MCV for each reference space was based on the length of line crossing each cell using randomly orientated lines. Since

MCV estimates were done on tissue sectioned in the coronal plane, rather than random planes, a small orientation bias could be present to the same degree for both autism and control cases.

Distinguishing Neurons From Microglia and Satellite Oligodendrocytes

Detailed morphological examination and characterization in 3D was performed in this study to distinguish neurons from microglia and satellite oligodendrocytes on Nissl-stained sections. Cell size alone was not sufficient because neurons possess much more cytoplasm and have distinct characteristics in their nucleus and processes. We estimate that, when randomly sampled, difficulty in judging whether a cell is a neuron, microglia, or satellite oligodendrocyte occurred in approximately 1 of 250 cells (which would contribute an error of <0.4% in neuron counts).

Statistical Analyses

Analyses were made using SPSS version 18.0.0 to assess within-case, between-case, and main and interaction effects of region (prefrontal, DL-PFC, M-PFC) and diagnosis (autism, control). An analysis of covariance (ANCOVA) was conducted using diagnosis as the independent variable, age and PMI as covariates, and brain data (ie, brain weight deviance expressed as percent difference from brain weight of age norms, neuron counts, microglia counts, neuron volume) as the dependent variables. With the neuron count models, diagnosis remained in the model as a significant factor; but age, PMI, and the interaction term were not significant. A second full model with PMI, diagnosis, and their interaction was evaluated. Diagnosis remained in the model as a significant factor, PMI and the interaction term were not significant. Pearson correlations were used to examine the relationships between neuron counts for DL-PFC and M-PFC, prefrontal neuron counts and brain weight deviance, and each of these variables and age. Using a best fit line

of the relationship between neuron counts and brain weight deviance in control children, neuron counts in each autistic child were used to predict the brain weight deviation from age-based norms. Independent samples *t* test with equal group variances were performed to test for differences in group means. Group variances were tested initially, and none were found to be significantly different. All tests of statistical significance were 2-sided. A *P* value of less than .05 was considered significant.

RESULTS

Diagnostic Characteristics

All autistic cases met criteria for autistic disorder on the 3 subscales of the Autism Diagnostic Interview-Revised (ADI-R) diagnostic assessment (Table 1). Scores on social and communication ADI-R scales ranged from less severe to more severe impairment. Intellectual ability ranged from having normal language and/or daily functional abilities to having little or no language comprehension and production and very impaired functional abilities (Table 1). No autism case had a diagnosis of Asperger syndrome or pervasive development disorder-not otherwise specified. One of the autistic children had received an autism diagnosis via the Autism Diagnostic Observation Schedule that had been administered when the child was still living (case 7, Table 1).

Clinical Characteristics

Except for 1 child in the control group, children in the autism and control groups were born full term and perinatal courses were unremarkable (TABLE 2). One 7-year-old in the autism group had a history of seizures and was being treated with medication. He had been diagnosed with a heart murmur at birth and had a fever 3 days after birth that required hospitalization. One 7-year-old in the control group received medication for hyperactivity. An 8-year-old in the autism group had rhabdomyosarcoma, received treatment including chemotherapy, and died of the

condition. Nonbrain fetal developmental defects were reported for 3 in the autistic group and 1 in the control group (Table 2). Most of the children died of acute global ischemic hypoxia (drowning, hanging, electrocution), 1 died in an automobile crash, 1 died of rhabdomyosarcoma, and 1 died suddenly of possible cardiac arrest (TABLE 3). Resuscitation was not in the medical history of any case. The prenatal, perinatal, medication, and medical histories and the causes of death among these 13 cases are not known to be associated with increases in neuronal numbers or brain size.

Neuropathological Characteristics

Gross examination of the brain showed no abnormalities in most autistic and control cases, according to medical examiner or neuropathology reports (eTable 3). In frontal lobes, neuropathology reports stated the presence of a single focal dysplasia associated with cortical thickening in 1 autistic case and a single ectopia in white matter and distortion of the normal radial orientation of neurons in superior-posterior cortex in another (Table 3). In the cerebellum, flocculonodular lobe dysplasia was reported in 4 of the 7 autistic cases (eTable 3). Pathologies of the cerebrum consistent with acute hypoxic ischemia were reported for 2 autistic cases and 2 control cases.

Brain Weight

The mean brain weight of the autistic children (1484 g; 95% CI, 1324-1644 g) was 2.4% greater than the mean brain weight reported for autistic 2- to 16-year-olds in the literature (N=18; 1449 g; 95% CI, 1182-1716 g) (eTable 4). This difference was not significant ($t_{1,23} = -0.5, P = .62$).

Brain weight in the autistic sample deviated from normative mean weight for age by 17.6% (95% CI, 10.2%-25.0%; $t_6 = 5.807; P = .001$), while control brains (1299 g; 95% CI, 1155-1442 g) deviated from age-based norms by 0.2% (95% CI, -8.7 to 9.1; $t_3 = 0.051; P = .96$) (FIGURE 2; TABLE 4; for age-based norms, Table 3). This autistic vs

control group difference was significant (group comparison, $P = .003$; Table 4).

Prefrontal Neuron Counts

Statistically significant differences in neuron counts in the PFC were found in the autistic children compared with controls (Table 4); counts for each autistic and control case in each region are shown in eTable 5. There were 79%

more neurons in DL-PFC in the autistic cases compared with the control cases (FIGURE 3A) and 29% more in M-PFC (Figure 3B). The mean DL-PFC count in the autistic children was 1.57 billion neurons (95% CI, 1.20-1.94) compared with a mean of 0.88 billion neurons (95% CI, 0.66-1.10) in control children ($P = .003$). The mean M-PFC count in the autistic group was 0.36 billion neurons (95% CI, 0.33-

Table 2. Clinical Characteristics of Autistic and Control Children in the Study

Case	Race/Ethnicity	Perinatal Condition	History of Medication	Seizures	Other Conditions
Autism					
1	White, Japanese, Native American, Hispanic	42 Weeks gestation; cesarean delivery; slight jaundice at birth	Perimortem dopamine	No	No
2	African American	Cesarean delivery	No	No	No
3	African American	No	No	No	No
4	White	No	Unspecified asthma medication	No	No
5	White	Heart murmur at birth; fever 3 d following birth that required hospitalization	Phenylbarbital, carbamazepine, albuterol	Yes	No
6	White	No	Chlorhexidine, nystatin, G-CSF, promethazine, dexamethasone, morphine, chemotherapy, divalproex	No	Syndactyly of the fingers and toes
7	White	Cesarean delivery; mother under "emotional distress" at time of birth	Pimozide (Orap)	No	Tourette syndrome, heart murmur at 8 mo of age
Control					
8	White	No	No	No	No
9	White Hispanic	No	Tacrolimus, immunosuppressants, unspecified antibiotics and antivirals	No	Gastroschisis; short bowel syndrome, renal failure, multivisceral transplantation, viral pneumonia
10	White Hispanic	≈ 25 Weeks gestation	Methylphenidate, clonidine	No	Hyperactive disorder
11	White	No	No	No	No
12	White	No	No	No	No
13	White	No	No	No	No

Abbreviation: G-CSF, granulocyte-colony stimulating factor.

PREFRONTAL CORTEX NEURON NUMBER AND SIZE IN AUTISM

0.40) compared with a mean of 0.28 billion neurons (95% CI, 0.23-0.34) in controls ($P=.009$). Together, these 2 subdivisions gave a total combined prefrontal neuron count that was 67%

greater in the autistic children (mean, 1.94 billion; 95% CI, 1.57-2.31) compared with controls (mean, 1.16 billion; 95% CI, 0.90-1.42; $P=.002$; Figure 3C). Significant group differ-

ences remained after controlling for PMI and age; ANCOVA model results are given in TABLE 5. Neither age nor PMI was a significant covariate in the models; however, diagnosis was sig-

Table 3. Neuropathology Characteristics and Postmortem Information on Autistic and Control Cases

Case	Age, y	Cause of Death	Postmortem Interval, h	Hemisphere	Brain Weight, g	Normative Mean Brain Weight for Age, g ^a	% Difference From Normative Mean Brain Weight for Age	Reported Frontal Cortex Neuropathology
Autism								
1	2	Drowning	4	Right	1328	1069	24	Single focal site in middle frontal gyrus with dysplasia, cortical thickening, loss of molecular layer, and thickening of layer 2; focal necrosis with gliosis and neurovascularization of layer 3 in frontal, temporal, parietal, and occipital cortices ^b
2	3	Drowning	12.5	Left	1389 ^c	1196	16	No report ^d
3	3	Drowning	15	Left	1330	1196	11	None ^e
4	4	Drowning	No records	Right	1280	1196	7	None ^b
5	7	Drowning	25	Right	1610	1361	18	Single 3 × 3 mm ectopia in periventricular frontal white matter lateral to anterior corpus callosum; distortion of radial cytoarchitecture in superior and posterior frontal cortices ^e
6	8	Rhabdomyosarcoma	22.2	Right	1570	1361	15	None ^e
7	16	Undetermined	47.9	Right	1880 ^f	1434	31	None ^b
Control								
8	2	Drowning	24	Left	1240	1069	16	None ^g
9	2	Respiratory insufficiency	14	Left	997	1069	-7	None ^g
10	7	Drowning	12	Right	1240	1361	-9	None ^g
11	13	Asphyxia by hanging	5	Right	1420	1434	-1	None ^g
12	14	Electrocution	20	Right	1464	1434	2	None ^g
13	16	Multiple injuries	16	Right	1440	1434	<1	None ^g

^aAdapted from Redcay and Courchesne.⁵

^bDetermined by coroner report and subsequent neuropathology reports.

^cInterpolated from brain volume from magnetic resonance imaging; differs from the 1130 g reported by the Autism Tissue Program.

^dCoroner and/or neuropathology reports not available through the Autism Tissue Program.

^eDetermined by neuropathology report only.

^fFresh brain weight at autopsy; differs from weight of 1990 g reported by the Autism Tissue Program. Parenchyma weight was estimated to be 1751 g, interpolated from in vivo magnetic resonance imaging brain volume at age 13 years.

^gDetermined by coroner report only.

nificant for DL-PFC and M-PFC regions and the total combined prefrontal regions.

eFigure 1 shows that these global increases in prefrontal neuron numbers were not apparent either at low or high magnification, and thus undetectable by neuropathology visual inspection and neuron density measurements without formal quantitative stereological procedures.

Prefrontal Neuron Counts and Brain Weight

FIGURE 4 plots the total prefrontal neuron counts as a function of percent difference of brain weight from age-based norms. The control group had a strong, significant positive linear correlation between counts and weight deviations ($r=0.949$; $P=.004$). Six of the 7 cases in the autistic group had neuron counts that met or exceeded the regression line of those in the control group, indicating that they had as many or more neurons than would be predicted from their large brain weights. The exception was a 7-year-old in the autistic group (Figure 4) who had a history of severe seizures. For the 6 cases in the autistic group without a confounding seizure disorder, the mean brain weight deviation predicted from their actual total prefrontal neuron counts was 29.4% beyond age-based norms.

Neuron Volume and Glia Counts

There were no significant differences in DL-PFC or M-PFC neuron sizes between groups. There were also no significant differences in glia counts for DL-PFC or M-PFC regions between groups (Table 4).

COMMENT

In this small, preliminary study, male children with autism had a mean 67% more prefrontal neurons than those in the control group. The excess was greater within DL-PFC than in M-PFC, a difference that parallels MRI volumetric data showing greater deviance in DL-PFC than M-PFC in living autistic toddlers.⁶ MRI studies show that

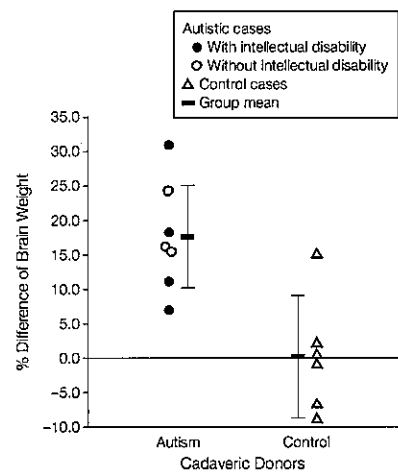
enlargement is not restricted to DL-PFC and M-PFC; whether increased neuron counts in autism extend beyond these 2 major prefrontal subdivisions to include other cortical areas remains to be determined.

The autistic group also had larger than average brain weight. In 6 of the 7 cases, neuron numbers equaled or exceeded predictions based on brain weight compared with controls. These data indicate that a pathological increase in neuron numbers may be a key contributor to brain overgrowth in autism. However, our data also illustrated a strong positive correlation between total neuron numbers and brain weight in the autistic cases. Thus, the autistic brains exhibited a substantial disturbance in the normal linear relationship between neuron quantity and overall brain weight. Neuron counts in the autistic children should have been accompanied by brain weights considerably larger than was observed, reaching 29.4% enlargement rather than the observed 17.6% enlargement. Thus, the size of the autistic brain, overlarge though it is, might actually underestimate the pathology of excess neuron numbers.

Because cortical neurons are not generated in postnatal life, this pathological increase in neuron numbers in autistic children indicates prenatal causes,

including unchecked proliferation, reduced apoptosis, or both.^{23,29-33} Proliferation of cortical neurons is exponential between 10 and 20 weeks gestation and normally results in a net overabundance of neurons by as much as 100%.³² In animal models, dysregulation of genetic mechanisms are known that cause an even greater neuron overabundance and lead to increased head, brain, and cortical size,^{34,35} as is found in young chil-

Figure 2. Difference in Brain Weight From Age-Based Norms in Autism vs Control Group



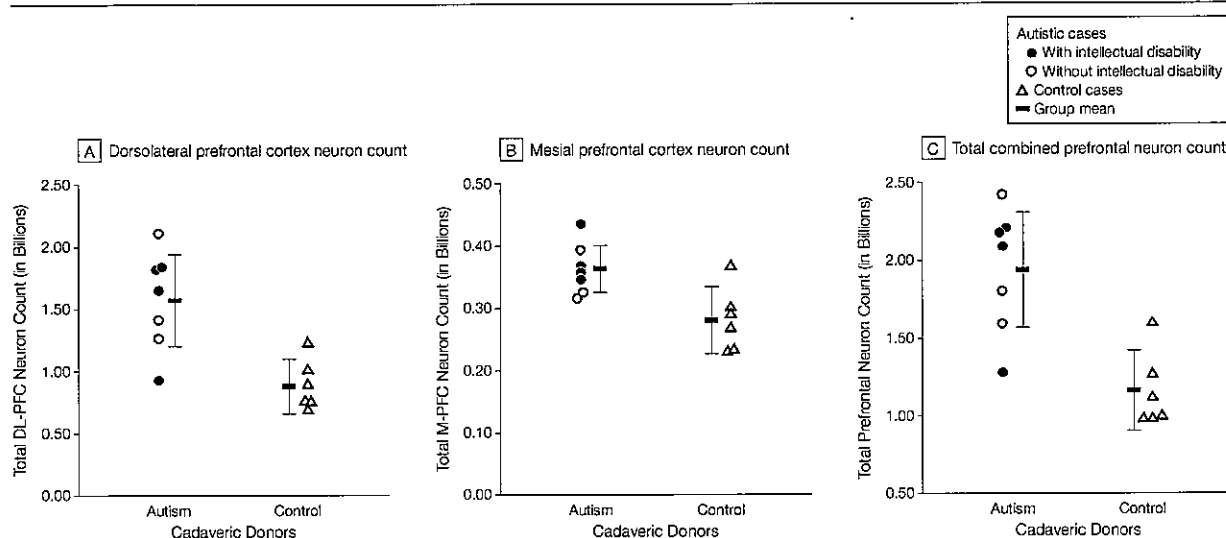
Brain weight in the autistic group deviated by 17.6% from the normative mean weight for age, while brain weight in controls was 0.2% greater than the normative mean for age. Error bars indicate 95% CIs. $P=.003$ for between-group comparison.

Table 4. Group Analyses of Brain Weight, Neuron Count and Size, and Glia Count

	Mean (SD)		t Value (df = 11)	P Value
	Control (n = 6)	Autism (n = 7)		
Postmortem brain weight, g	1299 (179)	1484 (216)	1.66	.12
Brain weight % difference from normative mean for age	0.2 (8.5)	17.6 (8.0)	3.81	.003
DL-PFC neuron count, billions	0.88 (0.21)	1.57 (0.40)	3.81	.003
M-PFC neuron count, billions	0.28 (0.05)	0.36 (0.04)	3.20	.009
Total PFC neuron count, billions ^a	1.16 (0.24)	1.94 (0.40)	4.12	.002
DL-PFC neuron size, μ^3	1337.12 (483.94)	1169.90 (244.81)	-0.81	.44
M-PFC neuron size, μ^3	1256.84 (352.76)	1127.81 (364.66)	-0.65	.53
DL-PFC glia count, billions ^b	0.36 (0.38) ^b	0.26 (0.28)	-0.51	.62
M-PFC glia count, billions	0.14 (0.13)	0.12 (0.20)	-0.28	.78

Abbreviations: DL-PFC, dorsolateral prefrontal cortex; M-PFC, mesial prefrontal cortex; PFC, prefrontal cortex.
^aTotal combined prefrontal neuron count equals DL-PFC count plus M-PFC count.
^bGlia counts in the DL-PFC for 1 case in the control group exceeded 3 standard deviations of the mean and was removed as an outlier. This child underwent repeated surgeries during early childhood, which may have altered glia numbers; however, his counts for M-PFC were near the group average.

Figure 3. Dorsolateral (DL-PFC) and Mesial Prefrontal Cortex (M-PFC) Neuron Counts in Autism vs Control Group Cases



Error bars indicate 95% CIs. For between-group comparisons, statistical tests were as follows: $P = .003$ for panel A, $P = .009$ for panel B, and $P = .002$ for panel C. Autistic case with lowest neuron count value in panels A and C had a seizure disorder, adverse perinatal medical conditions, and intellectual disability.

Table 5. Analysis of Covariance Tests

Controlling for Age	Group		Age, y	
	t Value	P Value	t Value	P Value
DL-PFC neuron count	3.39	.007	-0.75	.47
M-PFC neuron count	2.954	.01	0.066	.95
Total PFC neuron count ^a	2.954	.01	0.066	.95
DL-PFC neuron size	-0.58	.57	0.64	.54
M-PFC neuron size	-0.43	.68	0.663	.52
DL-PFC glia count ^b	-0.22	.84	0.606	.56
M-PFC glia count	-0.32	.76	-0.207	.84

Controlling for PMI	Group		PMI	
	t Value	P Value	t Value	P Value
DL-PFC neuron count	3.19	.01	-0.29	.78
M-PFC neuron count	2.69	.03	1.83	.1
Total PFC neuron count ^a	3.39	.008	-0.06	.95
DL-PFC neuron size	-0.83	.43	0.5	.63
M-PFC neuron size	-0.75	.47	0.318	.76
DL-PFC glia count ^b	-0.13	.9	-0.859	.42
M-PFC glia count	0.41	.69	-2.114	.06

Abbreviations: DL-PFC, dorsolateral prefrontal cortex; M-PFC, mesial prefrontal cortex; PMI, postmortem interval.
^aTotal combined prefrontal neuron count = DL-PFC count plus M-PFC count.
^bGlia counts for DL-PFC for 1 case in the control group exceeded 3 standard deviations of the mean and was removed as an outlier. This child had had repeated surgeries during early childhood, which may have altered glia numbers; however, his counts for M-PFC were near the group average

dren with autism.¹⁻¹² Functional analyses of genes located within copy number variation regions in autism also raise the possibility of dysregulation of proliferation during development.³⁶

Apoptotic mechanisms during the third trimester and early postnatal life normally remove subplate neurons, which comprise about half the neurons produced in the second trimester.³⁷

A failure of that key early developmental process could also create a pathological excess of cortical neurons. A failure of subplate apoptosis might additionally indicate abnormal development of the subplate itself. The subplate plays a critical role in the maturation of layer 4 inhibitory functioning as well as in the early stages of thalamocortical and corticocortical connectivity development.^{37,38} Reduced inhibitory functioning and defects of functional and structural connectivity are characteristic of autism, but the causes have remained elusive. The possibility of abnormal development of the subplate in autism merits investigation.

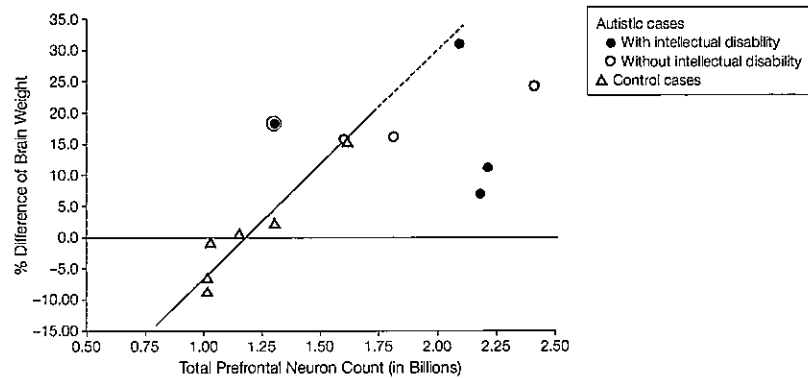
Future studies of neuron numbers and underlying molecular and genetic mechanisms in autism face many limitations, as encountered in the present study. For example, the sample of post-mortem tissue from children with autism—all that were available at the time of the study—was small. Despite the small sample size, evidence of excess neuron numbers in our autistic cases was statistically robust and occurred in cases with varying characteristics, such

as with less severe and more severe autistic symptoms, and with and without intellectual disability. None of the causes of death for autistic cases in this study produce an increase in postmortem brain weight³⁹ or neuron numbers. Most of the autistic and control children died of acute global ischemic hypoxia. Nearly every autistic and control case came from a full-term pregnancy. A history of medication and adverse medical conditions was not present in most of the cases, particularly for the 4 youngest autistic cases, each of whom had substantial excess neuron counts. Conversely, the lowest prefrontal neuron number in the autism group was found in a 7-year-old boy with a seizure disorder, which may explain why he had fewer counts than other autistic children. The potential effect of seizures on cellular and molecular measures in autism is important to investigate further.

Our sample of autistic children was not large enough to statistically examine brain-behavior relationships. Future studies with many more cases of autistic children might reveal important relationships between neuron counts and symptom severity or intellectual ability. Also, our sample of autistic cases had brain weights typical of brain weights in larger postmortem samples of autistic children. The 1 autistic child with a brain size within the 95% CI of controls had among the greatest prefrontal neuron counts in the study, which raises the question of whether excess prefrontal neuron counts may be present in other autistic children who have near normal or smaller brain sizes.

The small sample of young control children cannot be viewed as representative of all healthy young children, but control cases were not chosen for any reason other than age, sex, availability of the required PFC sections, absence of neurological or mental illness, and absence of treatment for cancer. Brain size in our control sample was typical for age, deviating by only 0.2% from expected mean weight for age. Also, prefrontal neuron counts in controls did

Figure 4. Prefrontal Neuron Counts as a Function of Percent Difference of Brain Weight From Age-Based Norms



Plot of total prefrontal neuron counts as a function of percent difference of brain weight from age-based norms for each study case. In control cases, the correlation between counts and % difference was $r=0.949$ ($P=.004$); the best-fit line for this is shown. Six of the 7 autistic children had neuron counts that met or exceeded the control line, indicating that they had equal to or more neurons than predicted from their large brain weight. An autistic boy with a seizure disorder was an exception in that he had fewer neurons than would be predicted for his brain weight (closed circle within circle). Three autistic cases without intellectual disability shown with extra open circle; each also met or exceeded the control regression line. The largest control brain from among approximately 11 000 brains (see Redcay and Courchesne⁵) had a percent difference from age-based norms of 22% where the solid best-fit line ends. The dashed line is an extrapolation because typical brains do not commonly achieve such size.

not vary with age, which is concordant with literature that cortical neurons are generated prenatally, not postnatally.^{23,29-33} It would be invaluable to study larger samples of autistic and control cases at a younger and narrower age range to confirm excess counts in autism at the youngest ages, as well as to study larger samples across a wider age range to identify patterns of age-related change in autism. It will be important to include female cases in future studies, as etiological mechanisms may be discordant between sexes. Whether female autistic patients also have excess prefrontal neuron numbers at young ages remains to be tested, but very few cases exist that have complete prefrontal sections.

To our knowledge, this study is the first direct quantitative test and confirmation of the theory that a pathological overabundance of neurons in critical brain regions is present at a young age in autism. Because cortical neurons are generated in prenatal, not postnatal life, pathological overabundance of neurons indicates early developmental disturbances in molecular and genetic mechanisms that govern prolif-

eration, cell cycle regulation, and apoptosis. Therefore, the finding has significance for understanding the etiological and neural development and functional origins of autism.

Author Contributions: Dr Courchesne had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Courchesne, Mouton, Semendeferi, Ahrens-Barbeau.

Acquisition of data: Courchesne, Mouton, Calhoun, Semendeferi, Ahrens-Barbeau, Barnes.

Analysis and interpretation of data: Courchesne, Mouton, Calhoun, Semendeferi, Ahrens-Barbeau, Hallet, Barnes, Pierce.

Drafting of the manuscript: Courchesne, Mouton, Barnes.

Critical revision of the manuscript for important intellectual content: Courchesne, Mouton, Calhoun, Semendeferi, Ahrens-Barbeau, Hallet, Pierce.

Statistical analysis: Mouton, Calhoun, Hallet, Pierce.

Obtained funding: Courchesne, Semendeferi.

Administrative, technical, or material support: Courchesne, Calhoun, Ahrens-Barbeau.

Study supervision: Courchesne, Mouton, Calhoun, Semendeferi, Ahrens-Barbeau.

Conflict of Interest Disclosures: All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Dr Calhoun is employed by and principal owner of Sinq Systems, a contract research organization that performed data collection and analysis for this study, and he is an applicant on a pending patent on file with the United States Patent and Trademark Office related to analysis of microscopic structure.

Funding/Support: This study was supported by Autism Speaks, Cure Autism Now, The Peter Emch Family Foundation, the Simons Foundation, The Thursday Club Juniors, and the University of Califor-

nia, San Diego-National Institutes of Health (UCSD-NIH) Autism Center of Excellence (P50-MH081755) awarded to Dr Courchesne and by the National Institute of Mental Health (NIMH) (MH076541-04) awarded to Dr Mouton. Tissue was provided by the National Institute of Child Health and Development (NICHD) Brain and Tissue Bank for Developmental Disorders under contracts N01-HD-4-3368 and N01-HD-4-3383, the Brain and Tissue Bank for Developmental Disorders, Autism Tissue Program, and direct donations to the Courchesne laboratory.

Role of the Sponsor: The funding organizations had no role in the design and conduct of the study; in the collection, management, analysis, and interpretation

of the data; or in the preparation, review, or approval of the manuscript.

Online-Only Material: The eAppendix, eTables 1-5, eFigure, and Author Audio Interview are available at <http://www.jama.com>.

Additional Contributions: We thank Chet Sherwood, PhD, George Washington University, for insight and review of prefrontal cytoarchitectonic features and regional borders; Muhammad Spocter, PhD, George Washington University, for insight and review of prefrontal cytoarchitectonic features and regional borders; Ronald Zielke, PhD, the Eunice Kennedy Shriver National Institute of Child Health & Human Development (NICHD) Brain and Tissue Bank for Developmental Disorders for facilitation of case acquisition; Jane Pickett, PhD, the Autism Tissue Program, for facilitation of case acquisition; Daniel Lightfoot, PhD, the Autism Tissue Program for facilitation of case acquisition; Patrick Hof, MD, at Mount Sinai School of Medicine, for facilitation of case acquisition; and Jerzy Wegiel, VMD-PhD, Institute for Basic Research, for facilitation of case acquisition. None of these individuals received compensation for their contributions to the study. We appreciate all parents who made the difficult choice to support brain research through the donation of brain tissue from their loved ones. This autism research is dedicated to the memory of Gail Erickson Courchesne, passionate and gifted musician and loving mother.

REFERENCES

- Courchesne E, Karns CM, Davis HR, et al. Unusual brain growth patterns in early life in patients with autistic disorder: an MRI study. *Neurology*. 2001;57(2):245-254.
- Courchesne E, Carper R, Akshoomoff N. Evidence of brain overgrowth in the first year of life in autism. *JAMA*. 2003;290(3):337-344.
- Carper RA, Moses P, Tighe ZD, Courchesne E. Cerebral lobes in autism: early hyperplasia and abnormal age effects. *Neuroimage*. 2002;16(4):1038-1051.
- Sparks BF, Friedman SD, Shaw DW, et al. Brain structural abnormalities in young children with autism spectrum disorder. *Neurology*. 2002;59(2):184-192.
- Redcay E, Courchesne E. When is the brain enlarged in autism? a meta-analysis of all brain size reports. *Biol Psychiatry*. 2005;58(1):1-9.
- Carper RA, Courchesne E. Localized enlargement of the frontal cortex in early autism. *Biol Psychiatry*. 2005;57(2):126-133.
- Courchesne E, Pierce K. Brain overgrowth in autism during a critical time in development: implications for frontal pyramidal neuron and interneuron development and connectivity. *Int J Dev Neurosci*. 2005;23(2-3):153-170.
- Hazlett HC, Poe M, Gerig G, et al. Magnetic resonance imaging and head circumference study of brain size in autism: birth through age 2 years. *Arch Gen Psychiatry*. 2005;62(12):1366-1376.
- Dawson G, Munson J, Webb SJ, Nalty T, Abbott R, Toth K. Rate of head growth decelerates and symptoms worsen in the second year of life in autism. *Biol Psychiatry*. 2007;61(4):458-464.
- Chawarska K, Campbell D, Chen L, Shic F, Klin A, Chang J. Early generalized overgrowth in boys with autism. *Arch Gen Psychiatry*. 2011;68(10):1021-1031.
- Schumann CM, Bloss CS, Barnes CC, et al. Longitudinal magnetic resonance imaging study of cortical development through early childhood in autism. *J Neurosci*. 2010;30(12):4419-4427.
- Hazlett HC, Poe MD, Gerig G, et al. Early brain overgrowth in autism associated with an increase in cortical surface area before age 2 years. *Arch Gen Psychiatry*. 2011;68(5):467-476.
- Courchesne E, Webb SJ, Schumann CM. From toddlers to adults: the changing landscape of the brain in autism. In: Amaral DG, Dawson G, Geschwind DH, eds. *Autism Spectrum Disorders*. New York, NY: Oxford University Press; 2011.
- Webb SJ, Dawson G, Bernier R, Panagiotides H. ERP evidence of atypical face processing in young children with autism. *J Autism Dev Disord*. 2006;36(7):881-890.
- Redcay E, Courchesne E. Deviant functional magnetic resonance imaging patterns of brain activity to speech in 2-3-year-old children with autism spectrum disorder. *Biol Psychiatry*. 2008;64(7):589-598.
- Santos M, Uppal N, Butti C, et al. von Economo neurons in autism: a stereologic study of the fronto-insular cortex in children. *Brain Res*. 2011;1380:206-217.
- Kennedy DP, Semendeferi K, Courchesne E. No reduction of spindle neuron number in fronto-insular cortex in autism. *Brain Cogn*. 2007;64(2):124-129.
- Braendgaard H, Cundersen HJG. The impact of recent stereological advances on quantitative studies of the nervous system. *J Neurosci Methods*. 1986;18(1-2):39-78.
- Mouton PR. *Unbiased Stereology: a Concise Guide*. Baltimore, MD: The Johns Hopkins University Press; 2011:20-21.
- Semendeferi K, Damasio H, Frank R, Van Hoesen GW. The evolution of the frontal lobes: a volumetric analysis based on three-dimensional reconstructions of magnetic resonance scans of human and ape brains. *J Hum Evol*. 1997;32(4):375-388.
- Rajkowska G, Goldman-Rakic PS. Cytoarchitectonic definition of prefrontal areas in the normal human cortex: I. remapping of areas 9 and 46 using quantitative criteria. *Cereb Cortex*. 1995;5(4):307-322.
- Bucy PC. *The Precentral Motor Cortex*. Champaign: University of Illinois Press; 1949.
- Samuelson GB, Larsen KB, Bogdanovic N, et al. The changing number of cells in the human fetal forebrain and its subdivisions: a stereological analysis. *Cereb Cortex*. 2003;13(2):115-122.
- West MJ. New stereological methods for counting neurons. *Neurobiol Aging*. 1993;14(4):275-285.
- Mouton PR. *Principles and Practices of Unbiased Stereology: an Introduction for Bioscientists*. Baltimore, MD: Johns Hopkins University Press; 2002.
- Mouton PR, Gordon M. Stereological and image analysis techniques for quantitative assessment of neurotoxicology. In: Harry CJ, Tilson HA, eds. *Neurotoxicology*. 3rd ed. Target Organ Toxicology Series. London, England: Informa Healthcare; 2010.
- Sterio DC. The unbiased estimation of number and sizes of arbitrary particles using the disector. *J Microsc*. 1984;134(pt 2):127-136.
- Jensen VEB, Cundersen HJG. The rotator. *J Microsc*. 1993;170(1):35-44 doi: 10.1111/j.1365-2818.1993.tb03321.x.
- Uyilings HBM, Malofeeva LI, Bogolepova IN, Jacobsen AM, Amunts K, Zilles K. No postnatal doubling of number of neurons in human Broca's areas (Brodmann areas 44 and 45)? a stereological study. *Neuroscience*. 2005;136(3):715-728.
- Bhardwaj RD, Curtis MA, Spalding KL, et al. Neocortical neurogenesis in humans is restricted to development. *Proc Natl Acad Sci USA*. 2006;103(33):12564-12568.
- Larsen CC, Bonde Larsen K, Bogdanovic N, et al. Total number of cells in the human newborn telencephalic wall. *Neuroscience*. 2006;139(3):999-1003.
- Gohlke JM, Griffith WC, Faustman EM. Computational models of neocortical neurogenesis and programmed cell death in the developing mouse, monkey, and human. *Cereb Cortex*. 2007;17(10):2433-2442.
- Rabinowicz T, de Courten-Myers GM, Petetot JM, Xi G, de los Reyes E. Human cortex development: estimates of neuronal numbers indicate major loss late during gestation. *J Neuropathol Exp Neurol*. 1996;55(3):320-328.
- Chenn A, Walsh CA. Regulation of cerebral cortex size by control of cell cycle exit in neural precursors. *Science*. 2002;297(5580):365-369.
- Kim WY, Wang X, Wu YW, et al. GSK-3 is a master regulator of neural progenitor homeostasis. *Nat Neurosci*. 2009;12(11):1390-1397.
- Pinto D, Pagnamenta AT, Klei L, et al. Functional impact of global rare copy number variation in autism spectrum disorders. *Nature*. 2010;466(7304):368-372.
- Kanold PO. Subplate neurons: crucial regulators of cortical development and plasticity. *Frontiers in Neuroanatomy*. 2009;3:1-9.
- Kostović I, Judas M. The development of the subplate and thalamocortical connections in the human foetal brain. *Acta Paediatr*. 2010;99(8):1119-1127.
- Hadley JA, Fowler DR. Organ weight effects of drowning and asphyxiation on the lungs, liver, brain, heart, kidneys, and spleen. *Forensic Sci Int*. 2003;133(3):190-196.