Supplementary Online Content

Courchesne E, Mouton PR, Calhoun ME; et al. Neuron number and size in prefrontal cortex of children with Autism. *JAMA.* 2011;306(18):2001-2010.

This supplementary material has been provided by the authors to give readers more information about their work.

eAppendix. Tissue Preparation; Anatomical Delineations of Mesial and Dorsolateral Prefrontal Cortex for Stereology Analyses; Comparison of Autistic Brain Weights in Current Study to Autistic Brain Weights in Current Study to Austic Brain Weights Reported in the Literature; Medical Examiner and Neuropathology Findings on Autism and Control Cases

1. Tissue Preparation

Brains were obtained from the National Institute of Child Health and Human Development (NICHD) University of Maryland Brain and Tissue Bank (UMB, BTB) and the Autism Tissue Program (ATP). Brains were prepared via three different pathways. Autistic and control cases were represented in each of these, as shown in eTable 1.

a. Switzer Pathway

Details of this procedure are described in Kennedy et al., 2007.[1] A summary description is as follows: Brain tissue was sectioned and stained by Dr. Robert Switzer of NeuroScience Associates (Knoxville, TN). The tissue was first cryoprotected in 20% glycerol-2% DMSO for one week. Then it was cast in a gelatin matrix and cured in formalin for 4 days. Next, the block containing the brain was rapidly frozen in a mixture of crushed dry ice and 2 methyl butane. The frozen block was then mounted on a large freezing stage and kept frozen with a collar of dry ice. Using a Lipshaw hydraulically driven microtome, the block was sectioned at 60–80 microns in the coronal plane. The sections were collected into standard buffered formalin, or 50% ethanol or antigen preserve (buffered ethylene glycol). Every 12th section was mounted on 3 x 5 inch slides and stained for Nissl substance with thionine. The staining sequence takes the sections/slides through defatting with chloroform/ether/Abs. ethanol, then HCL/EtOH, alcohol rinses, water and then thionine in acetate buffer at pH 4.5. The slides were rinsed in water, differentiated in alcohols and 95% EtOH acidified with acetic acid, further alcohol rinsed, dehydrated and cleared in xylene. The slides were coverslipped with Permount.

b. Celloidin Pathway

This processing pathway is based upon the methods described by Heinsen et al., 2000 [2]; a detailed summary of the process is available on the ATP portal (https://atpportal.org). Hemispheres processed according to this method are fixed for a minimum of 3 weeks in 10% buffered formalin and subsequently rinsed in increasing concentrations of ethyl alcohol. The entire hemisphere is then embedded in celloidin and hardened for 2.5 weeks while being exposed to chloroform vapors. Once hardened, the hemisphere is coronally cut into six series (two series at a thickness of 200u, and 4 series at a thickness of 50u). The serial sections are stored in 70% ethanol until they are Cresyl Violet stained and mounted on glass slides then cover-slipped.

c. Polyethylene Glycol Embedding (PEG) Pathway

In 2006, the ATP adopted the PEG protocol to process tissue. In the PEG pathway, hemispheres are cut coronally into 30mm slabs. These slabs are then infiltrated with increasing concentrations of polyethylne glycol and eventually embedded in fresh polyethylyne glycol. The embedded slabs are stored at 4°C and then cut into 50µm-thick sections. Tissue sections are stored in 70% ethyl alcohol until they are stained and mounted on glass slides.

2. Anatomical Delineations of Mesial and Dorsolateral Prefrontal Cortex for Stereology Analyses

Anatomical delineations of the mesial prefrontal (M-PFC) and dorsolateral prefrontal (DL-PFC) cortical regions throughout their rostrocaudal extent were based on previous definitions [3,4]. Delineation of the boundaries between DL-PFC and M-PFC used the terminology of the Atlas of the Human Brain [5], which correspond to the following Brodmann areas: lateral (44, 45, 46, and 47); mesial (12, 24, 25, 32 and 33); and the dorsomesial boundary (8, 9, 10 and 11).

In brief, M-PFC included the entire mesial surface of one hemisphere starting at the most rostral section and extending caudally until the paracentral lobule (PCL) had replaced M-PFC dorsally, the cingulate (CG) at mid dorsoventral levels, and the subcallosal area most ventrally. The M-PFC thus included the mesial portion of the superior frontal and gyrus rectus, and the inferior and superior rostral gyrus. The most rostral sections also included mesial portions of the frontopolar gyri.

For DL-PFC, the regions included were the lateral portion of the superior frontal gyrus, the middle frontal gyrus the inferior frontal gyrus, and the frontal operculum. The most rostral sections also included lateral portions of the frontopolar gyri. The dorsolateral surface was thus included starting rostrally and extending to a ventral termination at orbital PFC regions followed caudally by the insula/insular gyrus, and, within this gross definition, precentral and postcentral gyri were excluded. To simplify border delineation at each transition, a straight line was drawn normal to the curvature of the cortical surface.

As these cases varied in intrinsic cytoarchitectonics (as would be expected given the range of ages and the potential for diagnosis-related differences), it was not possible to apply a single criterion across all cases. Generally speaking, without full reconstruction, the unambiguous identification of individual sulci is not feasible on histological sections, and cortical regions also often transcend these boundaries, and thus the inclusion of cytoarchitectonic criteria is both necessary and useful. In some cases however, particularly when many rostro-caudal levels were available and other criteria were only suggestive, sulci were followed over multiple levels and used for definition. The following lists the various transitions/borders, and the most prominent features typically used to differentiate:

a) In the case of the lateral-orbital frontal cortex transition at rostral levels, because the transition is from two similar frontal cortex regions, the most reliable criteria are gross anatomical features, and included the fronto-marginal, lateral-orbital, and lateral sulci.

b) In the case of mesial cortex-cingulate transitions, the gross anatomy (shape of cingulate gyrus, presence of deep cingulate sulci) provided the initial primary criteria, followed by granularity of layer IV, the clarity of the IV/V transition, and the density of layer II. In some cases the cytoarchitectonics indicated cingulate extending slightly beyond or not quite reaching the bottom of the cingulate sulcus, but in the vast majority of cases, these criteria were in sync. The SCA ventrally had both clear cytoarchitectonic differences from the gyrus rectus, and differences in shape/curvature, and the first sulcus below the genu of the corpus callosum generally corresponded to the end of the cingulate.

c) The frontal operculum always started mesially at the insula border with the most mesial-dorsal inflection of the circular insular sulcus, and continued laterally to the precentral gyrus transition, which was marked by clear cytoarchitectonic features.

d) The border of frontal regions with the precentral gyrus was the most complex/variable. Identifying the border typically involved identifying a set of criteria specialized for each case that included identifying the cytoarchitectonics in the order below for regions that were clearly still frontal and comparing that to regions that were clearly not (e.g., more caudal parts of the precentral gyrus). The criteria were then mapped across sections and around the cortical layers until all criteria indicated the border location. The criteria were generally used in the following order:

   i. granularity of layer IV
   ii. packing density of cortical columns
   iii. cortical thickness
   iv. white-matter/layerVI transition
   v. layer IV/V transition
   vi. layer II thickness

The presence of Betz cells was only unambiguous in a minority of cases but when present would be among the primary criteria.

eFigure 1 shows examples of prefrontal cortex at low- and high-magnification. Images of DL-PFC and M-PFC are from a 3 year-old child with autism (case 3) compared with a 2 year-old control (case 8) and a 16 year-old boy with autism (case 7) compared with a 16 year-old control (case 13). The figure shows that global increases in prefrontal neuron numbers are not always apparent either at low or high magnification, necessitating the use of blinded quantitative stereology of the entirety of cortical regions of interest in autism, as done in the present study.

3. Comparison of Autistic Brain Weights in Current Study to Autistic Brain Weights Reported in the Literature

Brain weights of the present sample of N=7 autistic 2 to 16 year old males were statistically compared to brain weights of N=18 autistic 2 to 16 year olds reported in the literature. All brain weights for the N=18 autistic cases were summarized and published in Redcay and Courchesne, 2005 [6], see eTable 2.

Brain weight in the present N=7 sample of autistic cases were not significantly different compared to the N=18 other 2 to 16 year old autistic cases (see eTable 3; 1484 gm vs 1449 gm; $t(1,23) = -0.5$, $p=0.619$). ANCOVA analyses using age as a covariate (eTable 3) were also non-significant for brain weight ($P=0.271$) as well as for weight expressed as a % difference from normative mean for age ($p=0.08$), using the same norms as in the analyses of brain weight differences between the N=7 autistic and N=6 controls in the present study.

4. Medical Examiner and Neuropathology Findings on Autism and Control Cases

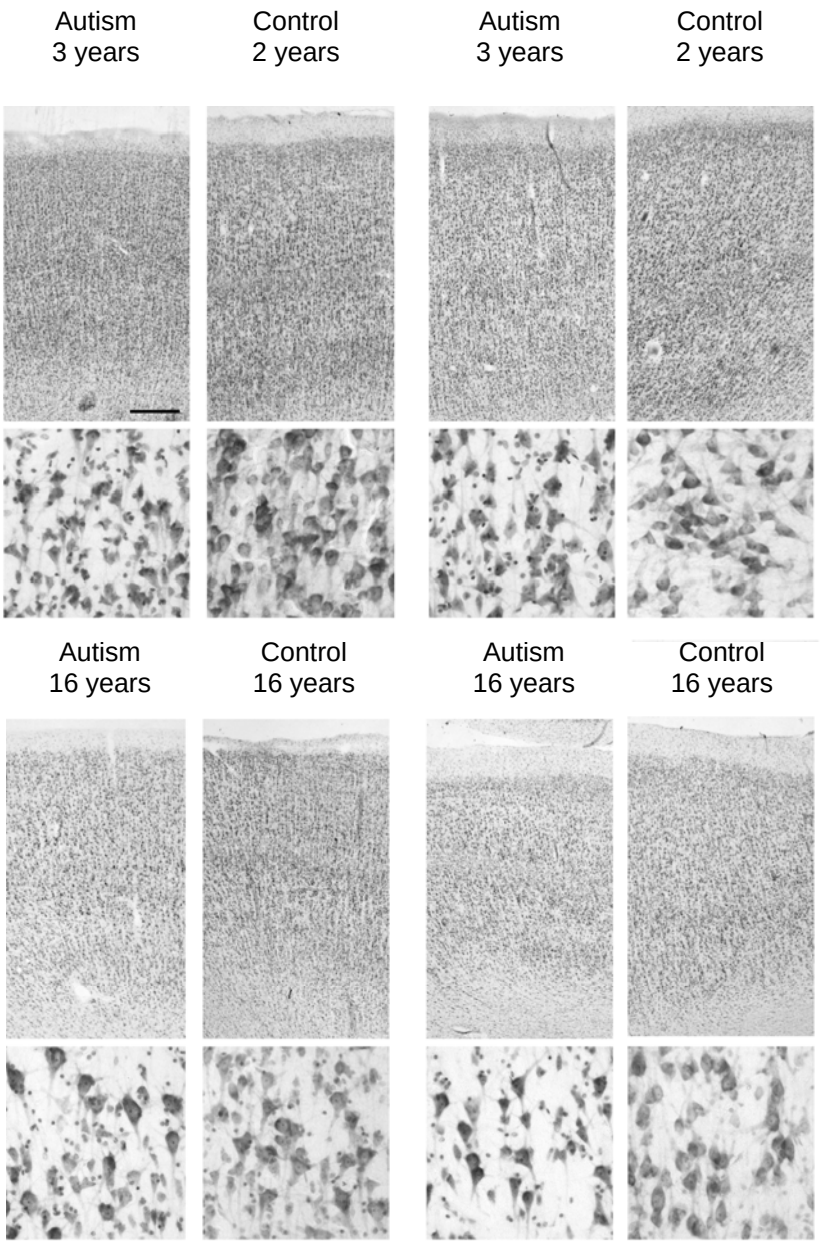
Findings in medical examiner and neuropathology reports are summarized for each of the autistic and control cases in eTable 4. Also included in eTable 4 are findings from Wegiel et al., 2010 [7] for autistic cases that overlap with the present study.

References

1. Kennedy DP, Semendeferi K, Courchesne E. No reduction of spindle neuron number in frontoinsular cortex in autism. *Brain Cogn*. 2007;64(2):124-129.

2. Heinsen H, Arzberger T, Schmitz C. Celloidin mounting (embedding without infiltration) - a new, simple and reliable method for producing serial sections of high thickness through complete human brains and its application to stereological and immunohistochemical investigations. *J Chem Neuroanat*. 2000;20(1):49-59.
3. Carper RA, Courchesne E. Localized enlargement of the frontal cortex in early autism. *Biol. Psychiatry*. 2005;57:126–133.
4. 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):375-88.
5. Mai JK, Paxinos G, Voss T. Atlas of the Human Brain, 3rd edition. 2008.
6. Redcay E, Courchesne E. When is the brain enlarged in autism? A meta-analysis of all brain size reports. *Biol Psychiatry*. 2005;58:1–9.
7. Wegiel J, Kuchna I, Nowicki K, et al. The Neuropathology of autism: defects of neurogenisis and neuronal migration and dysplastic changes. *Acta Neuropathol*. 2010;119: 755-770.

eFigure. Prefontal Cortex at High and Low Magnification in Autism and Control Cases



Objectives: Nikon Plan Apo 2x/0.1NA and 20x/0.7NA. Microfire camera coupled to Nikon Eclipse 90i microscope. Original images 70 pix = 250 mu for 2x, and 70 pix = 25 mu for 20x. Image resolution reduced 50% in each dimension . Scale bar 0.5mm for each low power and 50µm for each high power image. Camera, light, and microscope settings constant across ages and regions for each set of images (i.e., low-power & high-power). High-power images taken from the center of layer V.

eTable 1. Postmortem Tissue Processing Pathways for Autistic and Control Cases

	Case	Sectioning Protocol	Cut thickness (μm)	Stain
Autism	1	PEG	100 [a]	Cresyl violet
	2	Switzer	80	Thionine/Nissl
	3	Switzer	80	Thionine/Nissl
	4	Celloidin	200	Cresyl violet
	5	Celloidin	200	Cresyl violet
	6	Celloidin	200	Cresyl violet
	7	Switzer	80	Thionine/Nissl
Control	8	Switzer	80	Thionine/Nissl
	9	PEG	50	Cresyl violet
	10	Celloidin	200	Cresyl violet
	11	PEG	50	Cresyl violet
	12	Celloidin	200	Cresyl violet
	13	Switzer	80	Thionine/Nissl

[a] In order to preserve pure tissue, in this case four consecutive sections were cut at 100μm and 12 at 50μm; the series used in the present study was cut entirely at 100μm

eTable 2. Postmortem Brain Weights of 2 to 16-Year-Old Autistic Children From the Literature (N=18), but not in the Present Study

Case	Age	Brain Weight	Reported in
1	4	1525	Redcay & Courchesne 2005
BTB3871	5	1360	Redcay & Courchesne 2005
BCH-AUT-89-3	5	1386	Redcay & Courchesne 2005
	5	1550	Redcay & Courchesne 2005
UMB1349	5	1620	Redcay & Courchesne 2005
BCH-AUT-91	6	1460	Redcay & Courchesne 2005
AN03407	7	1575	Redcay & Courchesne 2005
BB3007	8	1525	Redcay & Courchesne 2005
BTB4231	8	1570	Redcay & Courchesne 2005
UMB0797	9	1175	Redcay & Courchesne 2005
UMB1315	9	1240	Redcay & Courchesne 2005
AN16641	9	1320	Redcay & Courchesne 2005
BCH-AUT-85	9	1454	Redcay & Courchesne 2005
BCH-AUT-87-3	12	1352	Redcay & Courchesne 2005
BB3003	12	1500	Redcay & Courchesne 2005
AN00754	13	1470	Redcay & Courchesne 2005; Wegiel 2010
AN00394	14	1615	Redcay & Courchesne 2005
AN02736	15	1390	Redcay & Courchesne 2005

eTable 3. Summary of Findings in Medical Examiner and Neuropathology Reports on Autistic Cases and Control Cases

Autism Cases	
1	**Gross Examination** (IBR)[a]**:** No abnormalities **Light Microscopy** (IBR)**:** *General observations* Hypoxic ischemia Necrosis due to cardiac arrest associated with drowning Wide spread neuronal loss observed in basal ganglia, entorhinal cortex, and brain stem *Cerebellum* Flocculonodular dysplasia Disorganization of the granule cell- Purkinje cell- and molecular layers Five islands of cells with altered features of cerebellar cortex in white matter
2	Autopsy and neuropathology reports not available
3	**Gross Examination** (ME)[b]**:** No abnormalities **Light Microscopy** (ME)**:** No abnormalities
4	**Gross Examination** (ME): No abnormalities **Light Microscopy** (Wegiel 2010): No abnormalities
5	**Gross examination** (IBR): No abnormalities **Light Microscopy**: *General observations* Hypoxic changes with moderate edema (ME) Two small heterotopias near frontal horn of lateral ventricle (Wegiel 2010) *Cerebellum* Flocculonodular dysplasia (Wegiel 2010)
6	**Gross Examination** (IBR)**:** No abnormalities **Light Microscopy:** *Cerebellum* (Wegiel 2010) Cortical dysplasia of vermis Flocculonodular dysplasia affecting most of lobe
7	**Gross Examination:** No abnormalities (ME) **Light Microscopy:** Neuronal heterotopias of thalamus (ME) *Cerebellum* (Wegiel 2010) Meningial cyst of caudal dorsal vermis Flocculonodular dysplasia affecting most of lobe

eTable 3. Summary of Findings in Medical Examiner and Neuropathology Reports on Autistic Cases and Control Cases

Control Cases	
8	**Gross Examination** (ME): No abnormalities **Light Microscopy** (ME): Neurons slightly shrunken in cerebral cortex and basal ganglia
9	**Gross Examination** (ME): Right cerebral cortex slightly thin Lateral ventricles slightly dilated Multifocal hemorrhages in right lateral occipital lobe **Light Microscopy** (ME): Some neurons with pyknotic nuclei in right cerebral cortex and basal ganglia Solitary cystic infarct in right occipital white matter; nodular dysplasia in left parietal cortex
10	**Gross Examination** (ME): No abnormalities **Light Microscopy:** Not available
11	**Gross Examination** (ME)**:** No abnormalities **Light Microscopy** (ME)**:** Not available
12	**Gross Examination** (ME): No abnormalities **Light Microscopy** (ME): Gliosis present in the peri-canal parenchyma.
13	**Gross Examination** (ME)**:** No abnormalities **Light Microscopy** (ME)**:** Not available

[a]IBR=Unpublished neuropathology report conducted by Institute for Basic Research in Developmental Disabilities.
[b]ME=Medical Examiner report- provided by University of Maryland Brain Bank.
Wegiel et al., 2010= Wegiel J, Kuchna I, Nowicki K, et al. The neuropathology of autism: defects of neurogenisis and neuronal migration and dysplastic changes. *Acta Neuropathol*. 2010; 119: 755-770.

eTable 4. Statistical Results of Postmortem Brain Weights in Study Autism Cases Compared With Brain Weights in 2 To 16 Year-Old Autism Cases From the Literature (Also Shows ANCOVA Results That Control for Age)

	Group				Age			
	Coefficient	**Std. Error**	**t-value**	**p-value**	**Coefficient**	**Std. Error**	**t-value**	**p-value**
Study vs Literature	-76.679	67.877	-1.130	.271	15.923	7.828	2.034	.054
% Difference in Brain Weight	-8.911	4.862	-1.833	.080	-.390	.561	-.695	.494

eTable 5. Neuron Counts in Prefrontal Cortex in Autism and Control Cases (in Billions)

	Case	Age	DL-PFC	M-PFC
Autism	1	2	2.10	0.32
	2	3	1.41	0.39
	3	3	1.84	0.37
	4	4	1.82	0.36
	5	7	0.93	0.35
	6	8	1.26	0.33
	7	16	1.65	0.44
Control	8	2	1.23	0.37
	9	2	0.75	0.23
	10	7	0.68	0.30
	11	13	0.71	0.29
	12	14	1.01	0.27
	13	16	0.89	0.23