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No reduction of spindle neuron number in frontoinsular cortex in autism

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Abstract

It has been suggested that spindle neurons, an evolutionarily unique type of neuron, might be involved in higher-order social, emotional, and cognitive functions. As such, it was hypothesized that these neurons may be particularly important to the pathophysiology of autism, a disease characterized in part by disruption of higher-order social and emotional processing. Therefore, we conducted the first stereological investigation of the number of spindle neurons in autism, using the optical fractionator technique. Our results did not provide evidence of a reduction in spindle neuron number in frontoinsular cortex in autism. However, this study provides the first quantitative stereological data on spindle neuron number in autism. Future postmortem studies with larger sample sizes will likely be critical in elucidating the spared and defective neural systems underlying the autistic phenotype.

Keywords: Von Economo Neurons; Postmortem; Stereology; Social cognition; Emotion

1. Introduction

Autism was first described in the scientific literature by Leo Kanner in 1943, who remarked at the similarities of social, communicative, and behavioral abnormalities in a group of children (Kanner, 1943). Since that initial description, numerous theories as to the underlying cause of the disorder have been put forth, ranging from early psychosocial explanations to our current understanding that autism is a biological disorder, rooted in abnormal neurodevelopment (for an interesting discussion of the history of autism, see (Schreibman, 2005)). Recent years have produced a wealth of information about brain abnormalities associated with the disorder (Belmonte, Cook, Anderson, Rubenstein, & Greenough, 2004; Courchesne, Redcay, & Kennedy, 2004; DiCicco-Bloom et al., 2006), although there are still enormous gaps that need to be filled, particularly in quantitative studies of the postmortem autistic brain (Palmen, van Engeland, Hof, & Schmitz, 2004). This is a glaring problem, since numerous hypotheses of exactly how, where, and which neural systems go awry in development depend heavily on data that often can only be obtained through histological studies.

The current experiment sought to test one such hypothesis—that there is a reduction in the number of spindle neurons in the autistic brain. Spindle neurons (also known as Von Economo Neurons (Allman, Watson, Tetreault, & Hakeem, 2005)), are large bipolar neurons found in cortical layer Vb in only two regions of the brain; frontoinsular cortex (FI) and rostral anterior cingulate cortex (rACC) (Nimchinsky, Vogt, Morrison, & Hof, 1995; von Economo, 1929). These neurons are evolutionarily unique, in that they are found in humans and great apes (bonobos, chimpanzees, orangutans, and gorillas), and have not been identified in 22 other primate species and 30 other

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mammals examined (Nimchinsky et al., 1999). The only known exception is that several species of whales possess these neurons, which the researchers suggest may be an example of convergent evolution (Hof & Van Der Gucht, 2006). Furthermore, they are found in greatest numbers in humans, being 5–40 times more common in humans than the great apes (Allman, Hakeem, Tetreault, & Semendeferi, 2003). Spindle neurons are four times larger than pyramidal neurons and their volume correlates with encephalization, being smallest in gorillas and greatest in humans. This correlation of spindle neuron volume with overall brain size is not seen in layer V pyramidal cells or the layer VI fusiform neurons.

Based on the functions normally attributed to the rACC and FI and the evolutionary uniqueness of spindle neurons, researchers have suggested that these neurons may play a key role in socioemotional and higher-order cognitive processing (Allman, Hakeem, & Watson, 2002; Allman, Hakeem, Erwin, Nimchinsky, & Hof, 2001; Allman et al., 2005; Nimchinsky et al., 1999), leading many to speculate that they may be dysfunctional in autism (Allman et al., 2005; Courchesne & Pierce, 2005; Mundy, 2003). Consistent with this speculation, functional abnormalities of the anterior insula (FI region) in autism have been found during processing of emotional expressions in faces (Hubl et al., 2003) and when making mental state inferences (Baron-Cohen et al., 1999), and there is evidence that FI might be overly functionally connected to the thalamus (Mizuno, Villalobos, Davies, Dahl, & Muller, 2006).

Furthermore, it was reported that in the typically-developing human brain, there are not full numbers of spindle neurons at birth, but rather, they emerge or differentiate over the first few years of life, although the precise developmental trajectory has yet to be fully worked out (Allman et al., 2005). This time period corresponds to the age when the autistic brain is undergoing rapid pathological overgrowth (Courchesne, Carper, & Akshoomoff, 2003; Redcay & Courchesne, 2005), particularly apparent in the frontal lobe (Carper, Moses, Tigue, & Courchesne, 2002). It has therefore been hypothesized that the protracted developmental time course of spindle neurons (and other large frontal lobe neurons) may make them particularly susceptible to early developmental derailment in the autistic brain (Courchesne & Pierce, 2005). Selective vulnerability of these neurons at later ages of life has recently been demonstrated in patients with frontotemporal dementia, a class of neurodegenerative diseases characterized in part by abnormal social and emotional functioning (Seeley et al., 2006).

Thus, due to the presumed role of spindle neurons in socioemotional processing, findings of functional abnormality in FI, and their protracted development that overlaps with brain growth abnormality in autism, it seems possible that these neurons might be affected in autism. Specifically, we hypothesized that there is a reduction in the number of spindle neurons in FI in the autistic brain.

2. Methods and materials

2.1. Subjects

The total number of spindle neurons in area FI was measured in four autism and seven control brain hemispheres, representing four and five individuals, respectively. Cases were matched for sex and hemisphere, and matched as close as possible for age (Table 1). All autism cases met criteria for autism on the ADI-R (Autism Diagnostic Interview-Revised (Lord, Rutter, & Le Couteur, 1994).

2.2. Tissue processing

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

Table 1
Case information

Diagnosis	Subject ID	Age	Sex	Hemi	Cause of death	Ethnicity	Seizures	Mental retardation	Brain weight	PMI (h)
Autism	BTB4029	3	M	L	Drowning	AA	N	N	1340	12.5
	B5223	15	M	R	Asphyxia	M	N	Y	1990	47.9
	CAL101	34	M	L	Adult respiratory distress syndrome, pneumonia	C	Y	Y	1367	16.5
	CAL104	41	M	L	Food aspiration	C	N	Y	1385	40
Control	BTB3958	2	M	L	N/A	N/A	N	N	N/A	24
	UMB1796	16.5	M	R	Car accident ⇒ cardiopulmonary arrest	C	N	N	1440	16
	H988	21	M	L & R	Gunshot	AA	N	N	1633	<18
	BTB3859	44	M	L	Acute myocardial infarction	C	N	N	1640	30
	SN207	75	M	L & R	Acute glomerulonephritis	C	N	N	1349	<18

PMI, postmortem interval; N/A, not available; AA, African American; C, Caucasian; M, Eastern Mediterranean.
BTB, University of Miami Brain and Tissue Bank; B, Harvard Brain Tissue Resource Center; CAL, Courchesne Autism Lab; UMB, University of Maryland Brain and Tissue Bank; H988 & SN207 Semendeferi Lab.

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 $3 \times 5''$ slides and then 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. This protocol was used for autism cases BTB4029, B5223, CAL101, and CAL104 and control cases BTB3958, UMB1796, and BTB3859.

For three cases (B5223, CAL101, BTB3958), the posterior portion of FI was obtained from the Amaral laboratory (M.I.N.D. Institute, UC Davis). These Nissl-stained coronal sections were 100 microns thick (for complete tissue processing details, see Schumann & Amaral, 2005, 2006). The anterior portion of FI (i.e., the remaining tissue) was sectioned according to the procedure described above.

Two cases (H988, SN207) were part of an existing brain collection and had been processed as follows. After formalin fixation, brains were embedded in paraffin, sectioned at 20 microns in the coronal plane, and every 15 section was stained with a modified Gallyas silver stain for neuronal perikarya (Merker, 1983); for additional processing details, see (Amunts, Malikovic, Mohlberg, Schormann, & Zilles, 2000).

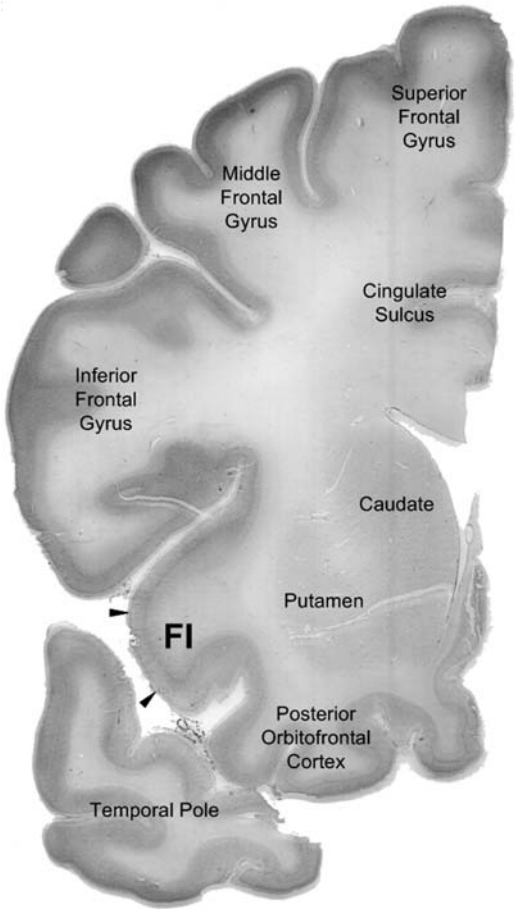

Fig. 1. Coronal section through the brain highlighting the location of frontoinsular (FI) cortex.

2.3. Area definition

The frontoinsular cortex is agranular cortex that makes up the anterior portion of the insula (von Economo & Koskinas, 1925; Mesulam & Mufson, 1982) (Fig. 1). Moving rostral to caudal, the transition from the posterior orbital frontal cortex (area FF) to the anterior insula (area FI) typically begins on the anterior wall of gyrus transverses insulae. Posteriorly, it is bounded by the lateral olfactory gyrus.

Two striking cytoarchitectural features unmistakably define area FI (von Economo & Koskinas, 1925). First, it is agranular cortex, with very few cells in layer II creating a discontinuous appearance, and a near complete or complete absence of layer IV. Second, layer Vb contains large bipolar spindle neurons, unique to FI and ACC, that make identification of the boundaries of FI unambiguous (for examples of spindle neurons, see Fig. 2). Once the rostral, caudal, medial, and lateral boundaries of FI were defined, the cortical layer containing spindle neurons (layer Vb) was outlined for all sampled slides.

2.4. Stereological probe

Stereology is a class of techniques that allows for the accurate estimation of parameters of interest in an unbiased, or assumption-free, manner. Further, the measurements are unaffected by differences in section thickness and processing. The stereological probe appropriate for counting numbers of neurons, known as the optical fractionator (West, 1993), was used according to standard protocol to determine the total number of spindle neurons in area FI.

Counting was carried out via a MicroBrightField system (MBF Bioscience, Williston, VT) connected to a Nikon E400 with a motorized stage and Dell PC with an Intel Pentium IV processor. As per standard stereological protocol (West, 1993), we systematically but randomly sampled from the entire rostral to caudal extent of area FI. The exact number of sections analyzed varied depending on the individual variability in the size and shape of FI, sectioning and staining interval, and the random sampling starting position (range: 9–25 sections, see Table 2).

While boundary definitions for FI were made using a 10× lens, counts were carried out using a 1.4 NA 60× oil objective with a matched 1.4 NA oil condenser, which produced a field of view of 160×125 μm on the computer video monitor. A randomly oriented scan grid of 200×200 μm squares was then overlaid on the defined region of interest (ROI). Within each scan grid square, a

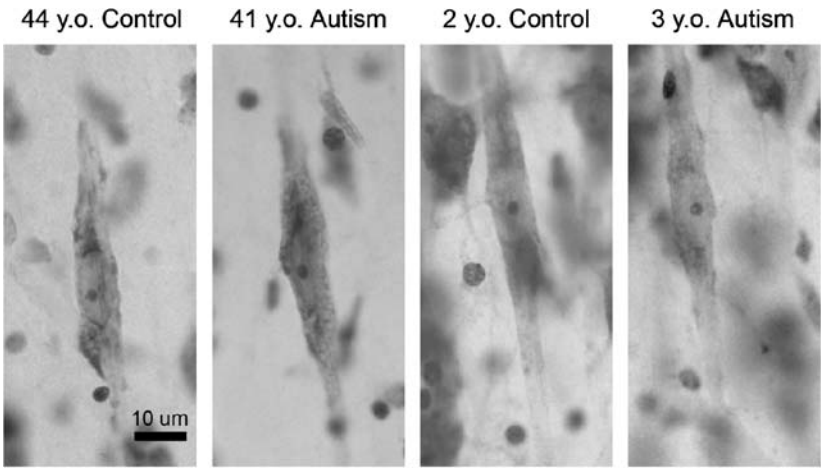

Fig. 2. Examples of representative spindle neurons of FI in older and younger control and autism brains. The same scale applies to all images.

Table 2
Stereological results for autism and control cases

Diagnosis	Subject ID	Hemi	Neurons counted	Sampling sites	Mean section thickness	Slides sampled	C.E.	Total neuron number
Autism	BTB4029	L	254	2553	15.2	10	0.07	32,935
	B5223	R	447	2850	19.2	12	<0.05	29,081
	CAL101	L	448	3516	20.2	17	<0.07	52,643*
	CAL104	L	175	1961	11.9	13	0.08	26,656
Control	BTB3958	L	281	2176	18	9	<0.07	20,788
	UMB1796	R	154	1498	16.3	11	0.08	21,365
	H988	L	125	741	21	12	0.09	29,990
	H988	R	121	1146	22	16	0.09	30,433
	BTB3859	L	334	1903	16.2	11	<0.05	41,484*
	SN207	L	118	1123	22.3	25	0.09	23,660
	SN207	R	136	1256	24	15	0.09	36,155

Neurons counted, number of neurons identified in the counting frames; Sampling sites, the number of counting frames visited across all sections for the case; Mean section thickness, average thickness of the tissue sampled from; Slides sampled, the number of slides sampled in the case; C.E., coefficient of error, or sampling error; Total neuron number, the estimated total number of neurons (see equation in Section 2).

* Average of two counts.

rectangular unbiased counting (sampling) frame of 125×100 μm was superimposed on the monitor simultaneously with the image. All sampling was restricted to the counting frame; thus, almost 1/3 of the ROI in each section was sampled (12,500 μm^2/40,000 μm^2).

The top and bottom of the tissue was not used for counting neurons, since these areas are known to be subject to significant sectioning artifacts (i.e., tissue thickness inhomogeneity, cell plucking, etc.) that can bias counts. Thus, at least a 4 μm top guard zone (measured from the first focal plane) and a minimum of a 2 μm bottom guard zone was implemented, such that neuron counts were made solely within the remaining volume of tissue that is more resistant to these edge-effect sectioning artifacts. This provided a sampling dissector height ranging from 6 to 10 μm (depending on post-processing section thickness), a sufficient thickness for resolving multiple optical planes under Kohler illumination and with the use of a 1.4 NA oil lens and a 1.4 NA oil condenser.

Spindle neurons were defined as elongated neurons in layer Vb that are larger than neighboring pyramidal cells and roughly symmetrical about the horizontal and vertical axis (see Fig. 2). When focusing down through the tissue, a spindle neuron was counted if it's nucleus came into focus at their widest and clearest point *within* the counting frame, or if it intersected the top, back or right inclusion planes. According to standard protocol, any nucleus that intersected the bottom, front, or left forbidden planes was excluded, even if it also intersected the inclusion planes (West, 1993). All counts were carried out blind to diagnosis, with the exception of two control cases (H988 and SN207) that were stained differently since they were part of a pre-existing collection.

Once the ROI had been sampled with the optical fractionator probe according to the criteria described above, an estimate of the total number of neurons (N) was calculated using the equation

$$N = Q \times 1/\mathrm{asf} \times 1/\mathrm{ssf} \times 1/\mathrm{hsf},$$

where Q = number of neurons counted, asf = area sampling fraction, ssf = section sampling fraction, and hsf = height sampling fraction. Thus, the estimated total

number of neurons is equal to the number counted multiplied by the inverse of the fraction of volume of the specimen sampled. For further stereological details of each case, see Table 2.

2.5. Sampling scheme

Since the definition of a spindle neuron is based only on neuron morphology and large size, additional steps were taken in order to ensure that consistent criteria were used for defining spindle neurons across cases. After boundary definitions of FI were made (see above), sampling proceeded in an across-case sequence, rather than the more typical within-case sequence, with autism and control cases randomly intermixed. Furthermore, pictures of counted spindle neurons were acquired such that upon resuming counting after a break, examples of neurons were previewed.

The goal of these two procedures was to limit the amount of drift in the definition of spindle neurons, increasing the reliability of counts. To ensure that this was achieved, two cases were recounted in their entirety. Our count-recount reliability, also performed blind to diagnosis and previous results, was greater than 85% for both cases, suggesting a high degree of stability in our spindle neuron counts.

3. Results

Spindle neurons were readily identifiable in both autism and control brains (Fig. 2). The number of spindle neurons was not significantly different between groups ($t(9) = 1.06$, $p = 0.32$). The mean number of spindle neurons in autism was 35,329 (SD = 11,829; SEM = 5914) and in controls was 29,125 (SD = 7791; SEM = 2945) (see Fig. 3), consistent with numbers previously reported for control brains (Allman et al., 2003). When the autism outlier (CAL101) was excluded from analysis (see Fig. 3), the average number of spindle neurons was nearly identical between groups (autism = 29,557; SD = 3166; SEM = 1828) ($t(8) = .09$, $p = 0.93$). Furthermore, in both cases that include both hemispheres (i.e., H988; SN207), the right FI has a greater number of spindle neurons than the left FI, a finding consistent with previous research on both humans and great apes (Allman et al., 2003). Additional stereological results are provided in Table 2.

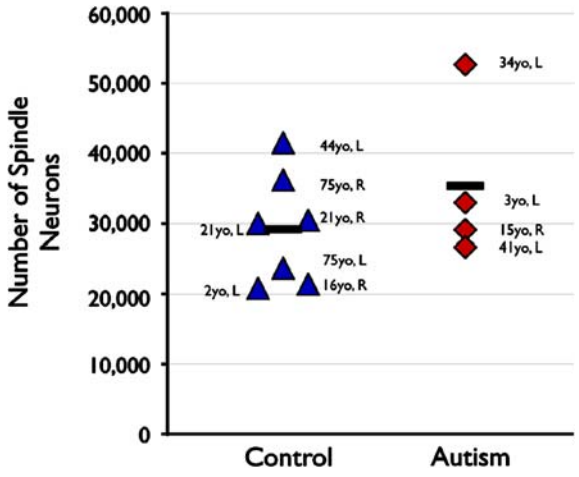

Fig. 3. Graph illustrating the number of spindle neurons in FI in control and autism brains. L, left hemisphere; R, right hemisphere. Black bars indicate the group mean.

Qualitatively, we observed no difference in the size of spindle neurons in control and autism cases at either the younger or older ages (see Fig. 2).

4. Discussion

Contrary to our initial hypothesis, we did not find a reduction in the number of spindle neurons in FI in the autistic brain. In fact, although not significant in the current sample, there may actually be a slight increase in spindle neuron numbers, possibly in only a subset of individuals with autism (Fig. 3; Table 2). This is consistent with a recent qualitative report (Allman et al., 2005) that observed an increased density of spindle neurons in FI in two autistic cases.

A limitation of the present study is the small number of autism cases measured. However, the scarcity of autism tissue precludes the inclusion of large numbers of brains in this and other histological studies of the autistic brain. Based on our present findings, a much larger sample size using stereological counting methods would be necessary in order to determine if there is a statistically significant increase in spindle neurons in autism.

Our results are not necessarily inconsistent with previous hypotheses of spindle neuron dysfunction in autism. We only measured one specific parameter of these neurons (i.e., their number), while other properties of these neurons might also affect their normal functioning. For instance, it was reported that spindle neurons are labeled with antibodies against vasopressin 1a, dopamine d3, and serotonin 2b receptors (Allman et al., 2005), so perhaps differences in receptor expression, binding affinity, or ligand availability would render these neurons dysfunctional. Or perhaps the dendritic architecture of these neurons is abnormal, or axons are improperly myelinated, or axons synapse on the wrong targets. Furthermore, we quantified spindle neuron number in FI only, whereas a difference might still be observed in the spindle neurons of the ACC.

Although our results failed to demonstrate a significant reduction in spindle neuron numbers in autism, our experiment and results are still important. First, our findings highlight the need for large sample sizes in postmortem studies of the autistic brain. In this study, the variation of neuron numbers in control and autistic brains is quite large, which would make it difficult to detect subtle group differences in small samples, even in well-matched groups. Further, only studies with larger sample sizes will be able to relate differences in anatomical measurements with clinical characteristics (e.g., seizure disorder, level of functioning, etc.). Second, there is a striking absence of studies of the postmortem autistic brain, especially those using quan-

titative stereological approaches (Martchek, Thevarkunnel, Bauman, Blatt, & Kemper, 2006; Schumann & Amaral, 2006). The field of autism research currently has numerous plausible brain-based theories that can potentially account for the autistic behavioral and cognitive phenotype, but a glaring lack of experimental data upon which to constrain, test, and refine these theories. Future quantitative studies of the postmortem autistic brain will likely prove instrumental in elucidating the neurodevelopmental processes that go awry in autism.

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