

# Microglial Activation and Increased Microglial Density Observed in the Dorsolateral Prefrontal Cortex in Autism

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**Background:** In the neurodevelopmental disorder autism, several neuroimmune abnormalities have been reported. However, it is unknown whether microglial somal volume or density are altered in the cortex and whether any alteration is associated with age or other potential covariates.

**Methods:** Microglia in sections from the dorsolateral prefrontal cortex of nonmacrencephalic male cases with autism ( $n = 13$ ) and control cases ( $n = 9$ ) were visualized via ionized calcium binding adapter molecule 1 immunohistochemistry. In addition to a neuropathological assessment, microglial cell density was stereologically estimated via optical fractionator and average somal volume was quantified via isotropic nucleator.

**Results:** Microglia appeared markedly activated in 5 of 13 cases with autism, including 2 of 3 under age 6, and marginally activated in an additional 4 of 13 cases. Morphological alterations included somal enlargement, process retraction and thickening, and extension of filopodia from processes. Average microglial somal volume was significantly increased in white matter ( $p = .013$ ), with a trend in gray matter ( $p = .098$ ). Microglial cell density was increased in gray matter ( $p = .002$ ). Seizure history did not influence any activation measure.

**Conclusions:** The activation profile described represents a neuropathological alteration in a sizeable fraction of cases with autism. Given its early presence, microglial activation may play a central role in the pathogenesis of autism in a substantial proportion of patients. Alternatively, activation may represent a response of the innate neuroimmune system to synaptic, neuronal, or neuronal network disturbances, or reflect genetic and/or environmental abnormalities impacting multiple cellular populations.

**Key Words:** Autism, cellular, immune, microglia, neuropathology, postmortem

Neuroimmune abnormalities have been linked to the pathogenesis of autism (1,2). These alterations include autoimmune abnormalities and upregulation of chemokines and cytokines such as interleukin-1 $\beta$ , interleukin-6 (IL-6), and tumor necrosis factor- $\alpha$  (3–11). Do these abnormalities reflect (or produce) neuroglial activation in the brains of patients with autism? Infrequent instances of gliosis were first reported in a subset of autism cases via a qualitative neuropathological assessment (12). Recently, a single study has qualitatively reported microglial and astroglial activation in the cerebellum and anterior cingulate and middle frontal gyri (7). A fractional area methodology found significant increases in human leukocyte antigen-DR<sup>+</sup> microglial staining in the cerebellum (7). These results provide evidence for microglial activation in autism but stop short of demonstrating quantifiable microglial abnormalities in the cortex, as well as determining the nature of these abnormalities. Somal volume increases are often observed during microglial activation, reflecting a shift toward an amoeboid morphology that is accompanied by retraction and thickening of

processes (13). Microglial density may also increase, reflecting either proliferation of resident microglia or increased trafficking of macrophages across a blood-brain barrier opened in response to signaling by cytokines, chemokines, and other immune mediators (13–16).

Additionally, the relationship of cortical microglial abnormalities to important covariates requires consideration. The first few years of life in autism are marked by brain overgrowth that is pronounced in frontal cortex, particularly dorsolateral prefrontal cortex (dlPFC) and medial prefrontal cortex (17–25), before receding by early adolescence (although macrencephaly is present in adulthood in a small fraction of patients [17,18]). Seizures may occur in anywhere from 5% to 44% of patients with autism (26). Despite these striking features, it is unknown whether microglial activation is present during early brain overgrowth or instead emerges later, and there has been no assessment of the relationship between neuroglial features and potential covariates like brain mass, as well as potential confounds like seizure or postmortem interval (PMI).

To address these questions, we assessed microglia in the dlPFC of the largest group of postmortem autism cases studied to date using an antibody to ionized calcium binding adapter molecule 1 (Iba-1), a calcium-binding adapter protein and marker of cells of monocytic lineage (27), which returns exceptional detail in both resting and activated microglia. In addition to a qualitative neuropathological assessment, we examined somal volume via isotropic nucleator and microglial density via stereological estimation using an optical fractionator. We independently assessed a macrencephalic adolescent male with a 1990 g brain, one of the largest ever reported (17), and a young female with autism. To further define the nature of microglial alteration, we examined colocalization of microglia with a cytokine recep-

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tor, interleukin-1 receptor type I (IL-1R1), that is rapidly upregulated in microglia during inflammatory responses (28).

## Method and Materials

### Tissue Acquisition

Fifteen autism cases and 9 control cases were examined (Table 1). Two of the autism cases, a young female and a severely macrocephalic male, were segregated from the quantitative assessment group a priori. The quantitative assessment autism group was comprised of all male cases with suitable formalin-fixed dlPFC tissue available from the national brain banks and the Courchesne laboratory collection. Twelve of the 15 autism cases were diagnosed with autism via the Autism Diagnostic Interview-Revised. The remaining three cases were diagnosed based on written descriptions from unscored Pre-Linguistic Autism Diagnostic Observation Schedule, Childhood Autism Rating Scale, or multiple neurology reports with detail sufficient to conclude the case met full DSM-IV criteria for autism. No Asperger's syndrome or pervasive developmental disorder—not otherwise specified cases were included. Five of the cases had been previously assessed for glial abnormality (7). The control group was comprised of all available adolescent and younger male cases, as well as a numerical match for six adults with autism over 20 years of age (two of which were subsequently removed due to concerns regarding comorbid conditions.) It was not possible to limit acquisition to a single consistent hemisphere of the brain.

### Tissue Sectioning

Tissue sectioning for eight cases ( $n = 5$  autism and  $n = 3$  control cases; Neuroscience Associates [NSA] processing in Table 1) was performed by Dr. Robert Switzer (Neuroscience Associates, Knoxville, Tennessee) as follows: whole hemispheres were cryoprotected in 20% glycerol–2% dimethyl sulfoxide (DMSO) for 1 week, then cast in a gelatin matrix that was cured for 4 days. The block containing the brain was rapidly frozen in a mixture of dry ice and 2% methylbutane. The frozen block was mounted and sectioned in the coronal plane at 80  $\mu\text{m}$ .

Tissue processing for 16 cases ( $n = 10$  autism and  $n = 6$  control cases; University of California, San Diego [UCSD] processing in Table 1) was performed by J.T.M. as follows: small blocks of formalin-fixed tissue were acquired from Brodmann area 9/46 of the dlPFC. The tissue was cryoprotected in 10% sucrose–.1% DMSO for 2 days, followed by 20% sucrose–.1% DMSO for 2 days, and then sectioned at 50  $\mu\text{m}$  on a freezing microtome.

### Immunohistochemistry

All examined tissue was processed via an Iba-1 immunohistochemistry protocol. For 16 UCSD cases, eight sections were processed per case with a distance of 300  $\mu\text{m}$  between sections. For eight NSA cases, eight individual gyri, isolated from four evenly spaced coronal sections covering the extent of the dlPFC, were processed per case.

All tissue was washed, slide mounted, and air dried for 2 nights. Immunohistochemistry was performed as follows: peroxidase activity was blocked via 30-minute exposure to 3% hydrogen peroxide in methanol. The slides were microwaved in simmering Antigen Retrieval Citra (Biogenex, San Ramon, California) for 10 minutes, followed by a 30-minute cooldown. The tissue was blocked and permeabilized with a solution of 5% normal goat serum and .1% Triton X-100 in tris-buffered saline (TBS) for 3 hours. Incubation with the rabbit polyclonal primary antibody to Iba-1 (Wako USA, Richmond, Virginia) was carried

out at 1:1000 concentration in .1% Triton X-100 in TBS for 40 hours at 4°C. The sections were then incubated in anti-rabbit secondary antibody prepared as described from avidin-biotin complex reagent kit (Vector Laboratories, Burlingame, California) for 2 hours, followed by 2 hours in avidin-biotin complex reagent. The sections were developed using 3-3' diaminobenzidine tetrahydrochloride (Vector Laboratories) as chromagen with a 12-minute exposure time. Finally, the sections were counterstained with hematoxylin/eosin (Vector Laboratories) for 7 minutes and dehydrated through a progressive series of 70%/95%/100%/100% ethanol (3 minutes each) and two 100% xylene (20 minutes) rinses; coverslipping was performed with Permount (Vector Laboratories).

For the assessment of Iba-1 and IL-1R1 colocalization, the tissue from the 15 nonmacrocephalic male UCSD cases ( $n = 9$  autism;  $n = 6$  control cases) was processed. The NSA cases were excluded due to modestly reduced antigen availability, which prevented consistent visualization of the IL-1R1 immunostaining. For each case, two sections were fully processed, along with two background correction sections, each of which excluded one of the primary antibodies. All tissue was washed, slide mounted, and air dried for 2 nights. The slides were microwaved in simmering Antigen Retrieval Citra for 10 minutes, followed by a 30-minute cooldown. The tissue was blocked and permeabilized with a solution of 5% normal goat serum and .1% Triton X-100 in TBS for 3 hours. Primary antibody incubation was carried out with anti-Iba-1 at 1:1000 and the mouse monoclonal primary antibody anti-IL-1R1 (Santa Cruz Biotechnology, Santa Cruz, California) at 1:25 in .1% Triton X-100 in TBS for 40 hours at 4°C. The sections were then incubated for 2 hours in two secondary antibodies: donkey anti-mouse Alexafluor 647 (Invitrogen, Carlsbad, California) at 1:2000 and donkey anti-rabbit Alexafluor 568 (Invitrogen) at 1:2000, treated with 1:1000 Hoechst solution for 5 minutes, then coverslipped with Vectastain (Vector Laboratories). IL-6, monocyte chemoattractant protein-1, transforming growth factor- $\beta$  receptor, and tumor necrosis factor- $\alpha$  receptor antibodies were similarly assessed but failed to return quantifiable staining. The Hoechst treatment failed to return any staining, perhaps due to extensive DNA damage caused by prolonged fixation.

### Data Acquisition

In both diagnostic groups, robust staining was observed that was highly specific for microglia and macrophages, as expected from prior immunostaining experiments performed by the manufacturer. Juxtavascular and perivascular microglia were distinguished from nonparenchymal Iba-1 positive perivascular macrophages on the basis of the rod-shaped morphology of the perivascular cells and their alignment with a blood vessel (visible via counterstain).

The Iba-1 light-level stain was assessed on a Nikon Eclipse 80i microscope (Nikon Instruments, Melville, New York) with a MicroBrightField cx9000 camera (MBF Bioscience, Williston, Vermont) via 100 $\times$  objective with a 1.4 numerical aperture lens in the presence of Köhler illumination. Gray matter (GM) was assessed from the pial surface to the boundary between layer VI and white matter. White matter (WM) was assessed from the boundary with layer VI to a line drawn between the fundus of neighboring sulci at the base of each gyrus.

A blinded qualitative neuropathological assessment of microglial morphology was conducted on a 0 (normal)/+ (moderate alteration)/++ (severe alteration) scale. Iba-1 positive somal volume and microglial cell density were estimated via the isotropic nucleator and optical disector features of Stereo Investigator (MBF Bioscience). In the density assessment, microglia

**Table 1.** Descriptive Information for All Cases

Subject Number	Diagnosis	Age	Methodology	Processing Technique	Hemisphere	Brain Mass (g)	PMI (h)	Fixation Time (mo)	Cause of Death
BTB-4021	Autism	3	LL	NSA	Left	1330	15	52	Drowning
BTB-4029	Autism	3	LL	NSA	Left	1130	13	51	Drowning
UMB-1349	Autism	5	LL, CL	UCSD	Right	1620	39	80	Drowning
UMB-1174 <sup>b</sup>	Autism	7	QA	UCSD	Right	1310	14	96	Seizure
UMB-4231 <sup>c</sup>	Autism	8	LL, CL	UCSD	Right	1570	12	41	Drowning
B-4925 <sup>d</sup>	Autism	9	LL, CL	UCSD	Right	1320	27	90	Seizure
UMB-797 <sup>e</sup>	Autism	9	LL, CL	UCSD	Right	1500	13	125	Drowning
BTB-2004 <sup>f</sup>	Autism	10	LL, CL	UCSD	Left	N/A	23	126	Drowning
BTB-3878 <sup>g</sup>	Autism	12	LL, CL	UCSD	Right	1630	23	60	Drowning
UMB-4899 <sup>h</sup>	Autism	14	LL, CL	UCSD	Right	N/A	9	16	Drowning
B-5223	Autism	16	QA	NSA	Right	1990	48	78	Undetermined, possible cardiac arrhythmia
BTB-3663 <sup>i</sup>	Autism	27	LL, CL	UCSD	Right	1420	30	84	Neuroleptic malignant syndrome
B-5173 <sup>j</sup>	Autism	30	LL, CL	UCSD	Right	1230	20	81	Gastrointestinal bleeding
CAL-101 <sup>k</sup>	Autism	34	LL	NSA	Left	1367	17	126	Adult respiratory distress syndrome
CAL-104 <sup>l</sup>	Autism	41	LL	NSA	Left	1385	N/A	74	Food aspiration
BTB-3958	Control	1	LL	NSA	Left	N/A	24	57	N/A
UMB-4670	Control	4	LL, CL	UCSD	Right	N/A	17	30	Commotio cordis
UMB-1796	Control	16	LL	NSA	Right	1440	16	53	Multiple injuries
UMB-1649	Control	20	LL, CL	UCSD	Right	N/A	22	62	N/A
B-6221	Control	22	LL, CL	UCSD	Right	1535	24	N/A	N/A
UMB-818	Control	27	LL, CL	UCSD	Right	N/A	10	121	Multiple injuries
B-5873	Control	28	LL, CL	UCSD	Right	1580	23	N/A	N/A
B-5813	Control	41	LL, CL	UCSD	Right	1815	27	N/A	N/A
BTB-3859	Control	44	LL	NSA	Right	1640	30	62	N/A

N/A, not available; PMI, postmortem interval; CL, interleukin-1 receptor type I colocalization quantification; LL, density and somal volume quantification of light-level ionized calcium binding adaptor molecule 1; QA, qualitative assessment of light-level ionized calcium binding adaptor molecule 1; NSA, Neuroscience Associates; UCSD, University of California-San Diego; 0, normal; 0/+, minor alteration; +, moderate alteration; ++, severe alteration.

<sup>a</sup>Case is not included in statistical analyses or ranking; the range indicates where the case would rank relative to the 13 autism cases included in statistical analysis.

<sup>b</sup>Medication history: Dilantin, Tegretol, Depakote, Valium, Lamictil.

<sup>c</sup>Medication history: Zyprexa, Reminyl, Adderall.

<sup>d</sup>Medication history: Fluvoxamine, Tegretol, Ritalin, Clonidine, Pondimin, Prozac, Luvox, Risperdal.

<sup>e</sup>Medication history: Desipramine.

<sup>f</sup>Medication history: Clonidine.

<sup>g</sup>Medication history: Ritalin, Clonidine.

<sup>h</sup>Medication history: Methadone, Trileptal, Zoloft, Clonidine, Melatonin.

<sup>i</sup>Medication history: Medical records unavailable.

<sup>j</sup>Medication history: Phenobarbital, Mysoline, Dilantin, Diamox, Zarotin, Tegretol, Diazepam, Clonazepam, Depokene, Tranxene, Cisapride, Valproic Acid.

<sup>k</sup>Medication history: Tegretol, Risperdal, Zyprexa, Melaril, Luvox, Depakote.

<sup>l</sup>Medication history: Tegretol, Epival, Cogentin, Zyprexa, Loxepac, Flurazepam, Synthroid, Dalmane, Stelazine, Nozinan, Rivotril, Chloral Hydrate, Largactil, Kemadrin, Haldol, Procyclidine, Ativan, Lithium, Risperdal, Anafranil, Sulfate Ferreux.

were sampled in a systematic random fashion. The sampling design achieved a Scheffer coefficient of error (CE) < .05 in all but four cases in gray matter; the remaining measurements had a CE < .07. In WM, all measurements had a CE < .07 other than one measurement with a CE < .10. The counting frame was set to achieve complete antibody penetration and was 10  $\mu\text{m}$  thick with an upper guard zone of 2  $\mu\text{m}$ . The inclusion criterion was the microglial cell nucleus, which was visible via counterstain. Somal volume was estimated for every microglial cell recorded during the density assessment via isotropic nucleator.

Ionized calcium binding adapter molecule 1/IL-1R1 colocalization was assessed via a 40 $\times$  objective on a Carl Zeiss Axiovert 40 inverted fluorescent microscope with deconvolution capabilities (Carl Zeiss, Thornwood, New York). Four fields of view, two per section, were randomly selected and captured for each case. Each field of view was 10  $\mu\text{m}$  thick and sampled every .5  $\mu\text{m}$ . Images were deconvolved via the nearest neighbor algorithm in the image analysis software Slidebook 4.2 (Intelligent Imaging, Santa Monica, California). Background correction was accomplished via capture of

the control slides in both channels immediately before capture of the field of view. The cutoff for a pixel being counted as signal was set at the maximum signal in the control slide in the empty channel opposite the staining with primary antibody, to control for both bleedthrough and background light. Colocalization was assessed via creation of a mask in the Iba-1 channel, which was then assessed for pixels intersecting the mask in the IL-1R1 channel. All objects three pixels in size or smaller were discarded to reduce background noise. The number of objects and total volume of objects per field were assessed.

### Statistical Analysis

All analyses were conducted with PASW 18.0 (SPSS, Inc., Chicago, Illinois). Thirteen autism and 9 control cases were assessed for group differences. The a priori excluded macrencephalic adolescent male case and young female case with autism were qualitatively assessed independently.

All potential covariates for which there was information available for 16 or more cases were examined. Insufficient data

**Table 1.** Continued

Seizure History	Clinical History	Morphology	Gray Matter Density Rank	White Matter Density Rank	Gray Matter Somal Volume Rank	White Matter Somal Volume Rank
No	None	0/+	4	5	5	6
N/A	None	+	1	3	11	11
No	Obesity, undescended testicle	+	11	12	2	2
Yes	Bilateral coloboma	++	1–2 <sup>a</sup>	10–11 <sup>a</sup>	>1 <sup>a</sup>	>1 <sup>a</sup>
No	None	0	7	4	6	10
Yes	Venous angioma in frontal lobe, chronic middle ear disease	0	12	13	10	8
No	Chronic migraine	++	6	6	4	3
No	Chronic ear infection	0/+	8	8	7	4
N/A	Moderate hypotonia	0	10	9	9	9
Yes	None	++	5	11	3	7
No	None	0	10–11 <sup>a</sup>	2–3 <sup>a</sup>	<13 <sup>a</sup>	<13 <sup>a</sup>
Yes	None reported	0/+	13	10	8	5
Yes	Scoliosis, gastric polyp	++	2	7	1	1
Yes	Pneumonia	0	9	1	12	12
Yes	None	0/+	3	2	13	13
No	None	0	1 C	3 C	7 C	7 C
No	None	0	3 C	7 C	6 C	2 C
No	None	0	2 C	1 C	8 C	8 C
No	None	0	5 C	6 C	4 C	4 C
No	None	0	6 C	2 C	1 C	3 C
No	None	0	4 C	5 C	2 C	1 C
No	None	0	8 C	9 C	3 C	5 C
No	None	0/+	9 C	8 C	5 C	6 C
No	None	0	7 C	4 C	9 C	9 C

were available to examine a correlation with brain pH or any cognitive measures. Due to a potential interaction with diagnosis, brain mass was not assessed as a covariate but was assessed independently for interaction effects. Due to a strong colinearity with processing technique [ $r_{pb}(22) = .79; p < .001$ ], hemisphere was not assessed as a covariate. Case age and tissue fixation time did not demonstrate a significant or trend interaction with any outcome measure and were excluded as covariates.

Therefore, Iba-1 microglial density and somal volume data were analyzed via analysis of covariance and partial correlation analyses with postmortem interval (PMI) and processing technique as covariates. Cases for which PMI information was not available were included in these analyses via projection of a value based on diagnostic group average. Effect sizes for a young case subgroup were calculated via Cohen's *d* at a confidence level of 95%. The impact of seizure was assessed via Student *t* test, comparing six cases with autism with medical records indicating a clinical history of seizures to five cases with records sufficient to judge a clinical history of seizure unlikely.

The Iba-1 and IL-1R1 colocalization data were analyzed via a nested analysis of variance that accounted for the collection of multiple fields of view from the same case.

## Results

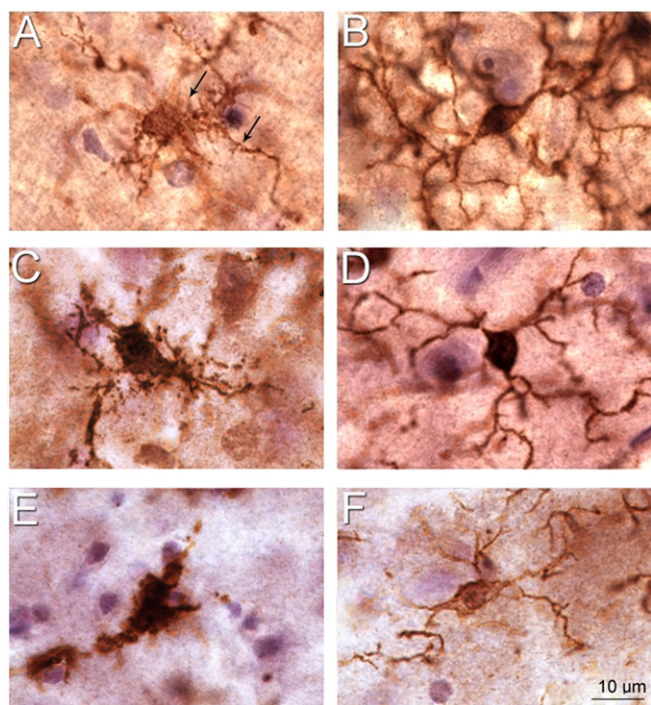
Microglial cell morphology was strongly altered (++) in 3 of 13 autism cases (Figure 1). Moderate alterations (+) were observed in 2 autism cases, both of which were under 6 years of age. Minor alterations (0/+) were observed in an additional 4 of 13 autism cases and 1 of 9 control cases; some of these alterations may reflect modest perimortem morphological changes. The alterations extended from the pial surface to deep white matter and were primarily characterized by somal enlargement and loss of definition alongside a reduction in process number, pro-

nounced thickening and shortening of processes, and extension of numerous filopodia from processes. Severely affected cases demonstrated a substantially amoeboid morphology in a few microglia, with a near absence of processes (Figure 1). Some morphologically resting microglia were present in all autism cases and were mixed relatively evenly among affected microglia (Figure S1 in Supplement 1). A macrocephalic (1990 g autopsy brain weight) adolescent autism case demonstrated resting microglial morphology (Figure 1). A young female autism case demonstrated severely altered microglial morphology (Figure 1).

Iba-1 positive microglial somal volume was increased in WM [ $F(1,18) = 7.59; p = .013$ ] in cases with autism relative to control cases, with a trend in GM [ $F(1,18) = 3.04; p = .098$ ] (Figure 2). Microglial cell density was increased in GM [ $F(1,18) = 13.59; p = .002$ ] but not WM [ $F(1,18) = 1.95; p = .18$ ] (Figure 2).

Gray matter microglial volume and WM microglial volume were positively correlated [ $r_{pb}(18) = .85; p < .001$ ] across all autism and control cases, as well as within cases with autism specifically [ $r_{pb}(9) = .91; p < .001$ ]. Gray matter microglial density was positively correlated with WM microglial volume [ $r_{pb}(18) = .52; p = .020$ ], and there was a strong trend toward correlation with GM microglial volume [ $r_{pb}(18) = .44; p = .055$ ] across all cases as a whole.

Given the neurodevelopmental features of autism, we separately analyzed the subgroup of young autism cases under 6 years of age. Effect sizes were very large for three out of four of our primary outcome measures (GM microglial somal volume, .94; WM microglial somal volume, .93; GM microglial density, .90; WM microglial density, .34). Alterations in all four quantitative measures were either close to or elevated beyond those observed in cases as a whole (GM microglial somal volume, 47% vs. 25%; WM microglial somal volume, 39% vs. 35%; GM microglial density, 22% vs. 28%; WM microglial

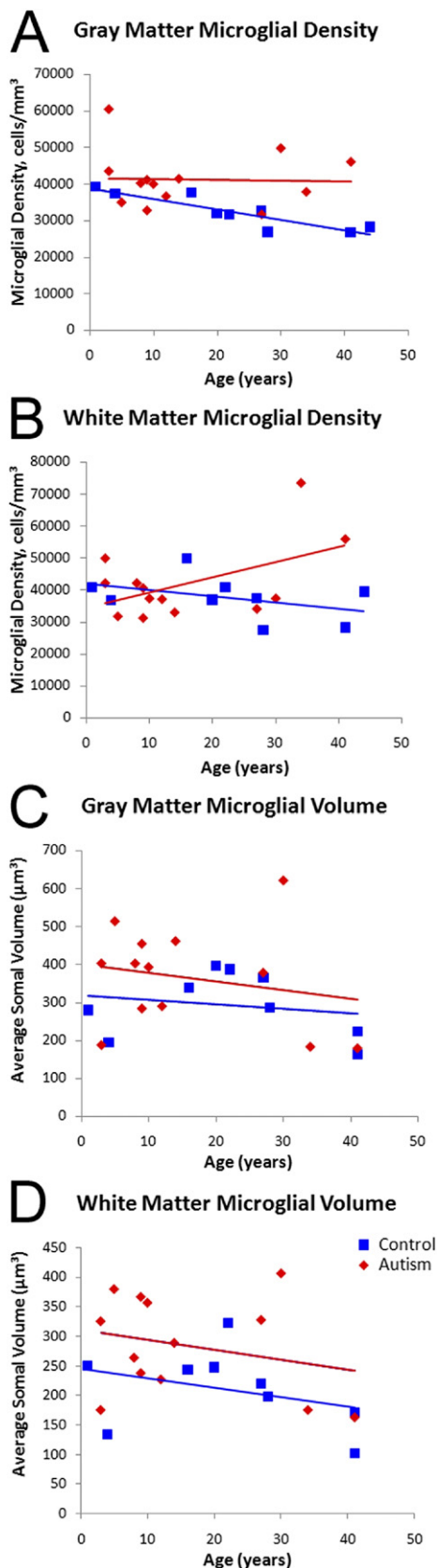


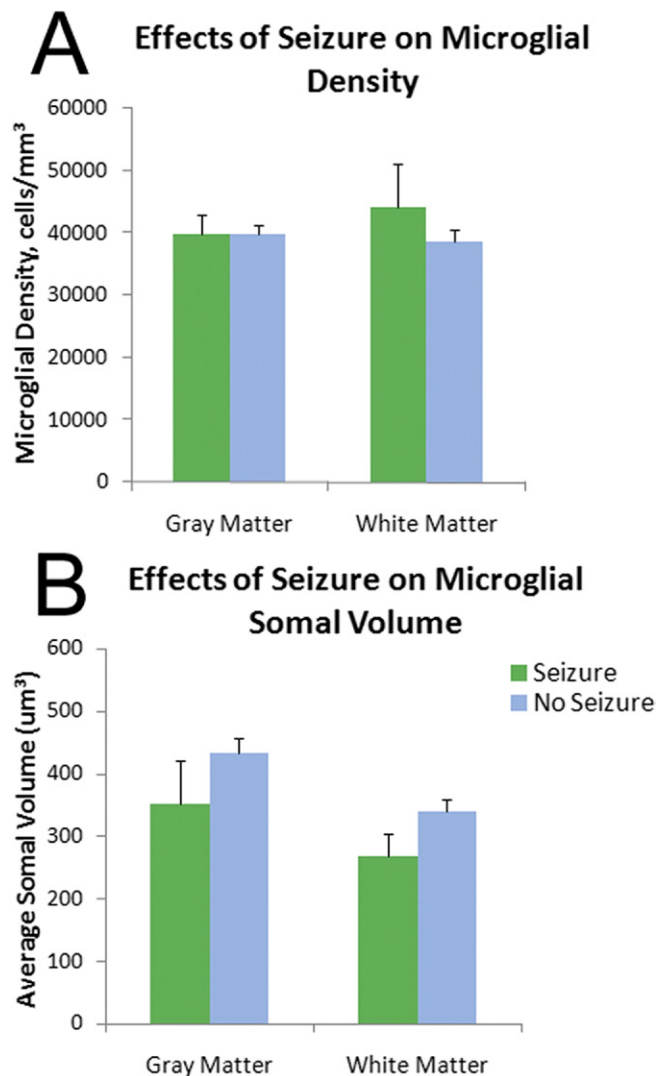
**Figure 1.** Qualitative alterations in ionized calcium binding adapter molecule 1 positive microglial morphology in autism. **(A)** Activated morphology in a 3-year-old case with autism (BTB-4021). Somal boundaries are expanded and poorly defined, and there is a relative reduction in number of processes along with a thickening of processes near the soma and an impression of extensive filopodia on many processes (indicated with arrows in A). **(B)** Resting morphology in a 4-year-old control case (UMB-4670). Note the small, clearly defined soma and extensive arbor of long, thin processes. **(C)** Activated morphology in the most morphologically affected adult with autism (B-5173). **(D)** Resting morphology in a control adult (B-6221). **(E)** Activated morphology in a 7-year-old female case with autism (UMB-1174) is marked by extensive somal swelling and thickening and loss of processes; this case was not included in our quantitative analyses due to gender but qualitatively was the most severely morphologically aberrant of our cases with autism. **(F)** Resting morphology in a 16-year-old autism case with severe macrocephaly and a history of seizure (B-5223).

density, 6% vs. 12%). However, sample sizes ( $n = 3$  autism,  $n = 2$  control cases) were insufficient to perform a formal analysis of group differences.

No significant differences were present in any microglial measures between seizure and nonseizure groups. The seizure group displayed reduced GM (−23%) and WM microglial somal volume (−27%), alongside equivalent GM microglial cell density (−4%) and nonsignificantly increased WM microglial cell density (+12%) (Figure 3). Seizure history was correlated with age [ $r(11) =$

**Figure 2.** Alterations in microglial cell density and somal volume in autism. **(A)** Microglial cell density in the gray matter is significantly increased in autism. There is significant heterogeneity; many cases overlap with control cases, while others show density increases of 60% to 80% relative to the control trend line. **(B)** Microglial cell density in white matter does not differ significantly between diagnostic groups. **(C)** There is a trend toward increased microglial somal volume in the gray matter. Up to threefold variability in average somal volume is present within the autism group in both volume measures, with some cases demonstrating a doubling in average somal volume relative to the control trend line. **(D)** Microglial somal volume in the white matter is significantly increased in autism.





**Figure 3.** No significant interaction is present between the presence of seizure and either microglial somal volume or density. **(A)** No consistent trend is apparent between microglial density and seizure in gray and white matter. **(B)** No significant differences in microglial volume are present between the seizure and seizure-free groups; trends suggest that microglial somal volume may, on average, actually be greater in the cases with no clinical history of seizure.

.85;  $p = .008$ ], and there was a trend toward correlation with brain mass [ $r(11) = -.68$ ;  $p = .052$ ].

Brain mass was negatively correlated with GM microglial density across all cases as a whole [ $r_{pb}(15) = -.60$ ;  $p = .002$ ] with a trend within autism cases specifically [ $r_{pb}(9) = -.64$ ;  $p = .094$ ]. Controlling for brain mass had little effect on the group difference in GM microglial density [ $F(1,18) = 13.64$ ;  $p = .002$ ]. Postmortem interval was negatively correlated with GM microglial density across cases as a whole [ $r(19) = -.58$ ;  $p = .029$ ], but this interaction did not reach significance within the autism [ $r(10) = -.46$ ;  $p = .13$ ] or control subgroups [ $r(6) = -.66$ ;  $p = .08$ ].

Tissue processing technique (NSA vs. UCSD) was correlated with GM microglial somal volume [ $r_{pb}(19) = .68$ ;  $p = .001$ ], WM microglial somal volume [ $r_{pb}(19) = .64$ ;  $p = .002$ ], and WM microglial density [ $r_{pb}(19) = -.70$ ;  $p < .001$ ] across all cases as a whole and also within autism cases specifically [ $r_{pb}(10) = .65$ ;  $p = .023$ ], [ $r_{pb}(10) = .58$ ;  $p = .049$ ], and [ $r_{pb}(10) = -.76$ ;  $p =$

.004], respectively, and control cases specifically [ $r_{pb}(6) = .87$ ;  $p = .006$ ], [ $r_{pb}(6) = .90$ ;  $p = .003$ ], and [ $r_{pb}(6) = -.76$ ;  $p = .028$ ], respectively.

To describe the pattern of alteration in finer detail, we generated histograms of GM microglial somal volume for each case (Figure 4). All cases demonstrated a relatively smooth, moderately skewed-right distribution of volumes. No control case demonstrated greater than 5% of microglia with a somal volume greater than  $1000 \mu\text{m}^3$  but one autism case had more than 17% of microglia greater than  $1000 \mu\text{m}^3$  in somal volume and an additional three autism cases had 5% to 8% of cells greater than  $1000 \mu\text{m}^3$  in volume (Figure 4). However, even in these most profoundly affected cases, some microglia displayed somal volumes at or below the control mean.

To examine whether microglial alterations reflected a prototypical acute inflammatory reaction, we performed a colocalization assessment of IL-1R1 with Iba-1 (Figure 5). No significant differences were present; nonsignificant trends indicated increased colocalization in control cases via both object number ( $p = .40$ ) and total volume ( $p = .09$ ) analyses.

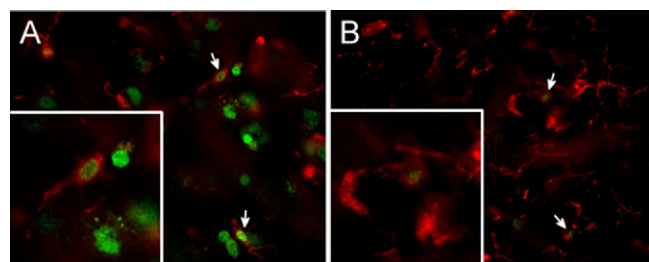
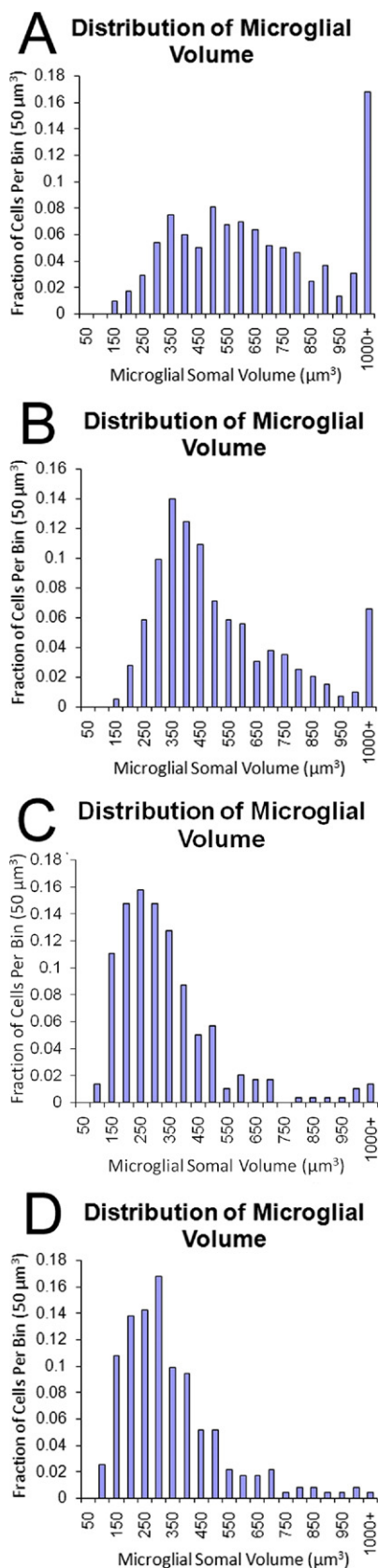
## Discussion

Moderate to strong alterations in Iba-1 positive microglial morphology indicative of activation (13,29) are present in 5 of 13 postmortem cases with autism, and mild alterations are present in an additional 4 of 13 cases. These alterations are reflected in a significant increase in average microglial somal volume in white matter and microglial density in gray matter, as well as a trend in microglial somal volume in gray matter. These observations appear to reflect a relatively frequent occurrence of cortical microglial activation in autism.

Of particular interest are the alterations present in two thirds of our youngest cases, during a period of early brain overgrowth in the disorder. Indeed, neither microglial somal volume nor density showed significant correlation with age in autism, suggesting long-running alteration that is in striking contrast with neuronal features examined in the same cases (Morgan *et al.*, unpublished data, 2009). The early presence of microglial activation indicates it may play a central pathogenic role in some patients with autism.

There was significant heterogeneity, both qualitative and quantitative, in the microglial abnormalities observed. Four of 13 of the quantitatively assessed autism cases demonstrated no alterations in microglial morphology. By contrast, 5 of 13 autism cases, as well as a young female autism case, demonstrated dramatic alterations, which produced quantifiable alterations as extreme as an 80% increase in density or doubling of average microglial somal volume relative to the age-corrected control mean. The variable presence of microglial activation suggests that future postmortem studies examine this feature for correlation with other cellular and genetic alterations. It should also be noted that while average microglial somal volume and gray matter microglial density are positively correlated, a few autism cases demonstrate marked alterations in one outcome measure but not the other. Thus, the features of microglial alteration in the disorder may be moderately heterogeneous.

No interaction was observed between seizures and any of our outcome measures, suggesting this commonly advanced explanation for neuroglial alteration is not responsible for the abnormalities reported here. Indeed, the trends suggest that patients with autism who display microglial alteration might be a different group than those with frequent seizures. We also observed a reduction in gray matter microglial density in autism cases with larger brains. One possible explanation is that brain growth moves microglia farther apart. Another explanation is that activation that produces mono-



**Figure 5.** Interleukin-1 receptor, type I (green) colocalization with ionized calcium binding adapter molecule 1 (red) after background correction. **(A)** A case with autism (B-5000). Instances of colocalization with ionized calcium binding adapter molecule 1 are indicated with arrows. Inset: 2× magnified view of the upper instance of colocalization. **(B)** A control case (UMB-818). Inset: 2× magnified view of the upper instance of colocalization. Despite the increased intensity of interleukin 1 receptor, type I staining in the case with autism, group trends were in the direction of more frequent colocalization and a greater total volume of colocalization in control cases.

cyte recruitment or proliferation results in degeneration that eventually reduces brain size. It may also be the case that etiologic profiles marked by increased monocyte recruitment are different from those that display aberrant brain growth. Our one autism case with severe macrocephaly (i.e., a 1990 g brain weight) demonstrated a lack of activation.

Several potential confounds remain. Medication history cannot be well controlled in our sample; however, moderate alterations (+) were present in an autism case with no reported medication history (UMB-1349). The most concerning potential confound, and one that we cannot statistically address due to group differences and a lack of control of medical records, is the cause of death, which was drowning in 8 of 13 autism cases. However, there is some reason to believe that the alterations we report are not attributable to drowning or other perimortem anoxic events. First, while morphological alterations are possible in a brief perimortem or postmortem period, it is unlikely that detectable increases in microglial density would be achieved. Indeed, there was a tendency toward reduced microglial density with increased PMI that may reflect a modest reduction in staining in the longest PMI cases. While Iba-1 staining intensity increases modestly in activated microglia (30), strong staining and fine detail were apparent in Iba-1 positive resting microglia in our samples. Second, there is no increase in microglial colocalization with a receptor, IL-1R1, typically upregulated in acute inflammatory reactions (28). The trend toward an increase in colocalization in control cases may also hint at downregulation of inflammatory signal receptors in a chronically activated system. It must be noted that substantial IL-1R1 staining was present in unidentified cells, which may have been astrocytes or neurons, and appeared to be substantially nuclear, a surprising finding given the primary function of IL-1R1 as a surface receptor (28).

The source of the observed microglial alterations remains uncertain. Activation might be triggered primarily environmentally, with

**Figure 4.** Histograms reveal relatively smooth distributions of microglial morphology in the gray matter in three cases with autism (**A,B,C**). The most severely affected cases still demonstrate a subset of microglia at normal somal volume. **(A)** Somal volume distribution in the most severely affected autism case (B-5173). **(B)** Somal volume distribution in an autism case ranked 2 to 4 in relative abnormality (UMB-797). **(C)** Somal volume distribution in an autism case demonstrating alteration in line with the control mean (B-4925). **(D)** Somal volume distribution in the control case closest to the mean of the control group (B-5813).

the response likely contingent on specific gene-environment interactions. In rodent models, maternal viral infection or prenatal intracerebral injection with virus can result in a wide range of behavioral and neurostructural abnormalities in the offspring (31,32). Alternately, autoantibodies to a diverse set of brain proteins have now been observed in both patients with autism and their mothers, although there is little consistency in the antigens (33–45) and infiltration of T-cells into the brain was not observed in a previous study (7). Indeed, chronic innate immune system activation might gradually produce autoimmune abnormalities via the occasional presentation of brain proteins as antigens (46).

Microglial activation might also represent an aberrant event during embryonic monocyte infiltration that may or may not also be reflected in astroglial and neuronal populations (17), given the largely or entirely separate developmental lineage of microglia (13). Alternatively, alterations might reflect an innate neuro-immune response to events in the brain such as excessive early neuron generation or aberrant development of neuronal connectivity. Finally, they may reflect genetic alterations directly affecting the innate immune system. Exploration of these possibilities will require examination of the other cellular populations of the brain in developmental postmortem tissue.

Regardless of the trigger, numerous secondary effects of microglial activation are possible. Microglial activation has been implicated in damage to multiple neural cell types in an array of clinical disorders (47–50). Reduced neuron numbers have been reported in older postmortem cases of autism in regions of early overgrowth and/or functional aberrancy (51,52), although it remains unknown whether neuron numbers are also altered in young autism cases. Additionally, frontal and parietal cortex and cerebellum in autism display reduced levels of anti-apoptotic factor Bcl-2 and increases in pro-apoptotic factor p53 (53–55). Qualitative alterations in the organization of neuronal populations in postmortem autism cases have been observed across many brain regions in several studies (7,12,56–62). The most consistently reported qualitative microstructural abnormality is loss of Purkinje neurons (7,12,59–62); this population is naturally culled during development via reactive oxygen species released from microglia, a process that is upregulated during activation (63). It must be noted, however, that any cell loss produced by microglial activation might be beneficial if, for example, there is early neuronal excess. In addition to affecting neuronal survival, microglia are now thought to play central roles in regulating synaptic development and function (64,65). Activated microglia may increase their production of growth factors such as brain-derived neurotrophic factor (66–68), which would adversely affect the development of neuronal connectivity.

In summary, our findings suggest that microglial activation may be present throughout the life span in many patients with autism, including at an early, developmentally critical age. To better understand the origin and effects of this phenomenon, it will be important to quantitatively determine whether microglial activation takes place alongside other cellular and neuroimmune abnormalities. Detailed, quantitative knowledge of microglial alteration in autism may substantially impact the search for mechanisms of pathogenesis, more reliable early identification tests, and treatments with greater efficacy.

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