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ORIGINAL PAPER

Microglia in the Cerebral Cortex in Autism

Nicole A. Tetreault · Atiya Y. Hakeem · Sue Jiang · Brian A. Williams · Elizabeth Allman · Barbara J. Wold · John M. Allman

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Abstract We immunocytochemically identified microglia in fronto-insular (FI) and visual cortex (VC) in autopsy brains of well-phenotyped subjects with autism and matched controls, and stereologically quantified the microglial densities. Densities were determined blind to phenotype using an optical fractionator probe. In FI, individuals with autism had significantly more microglia compared to controls ($p = 0.02$). One such subject had a microglial density in FI within the control range and was also an outlier behaviorally with respect to other subjects with autism. In VC, microglial densities were also significantly greater in individuals with autism versus controls ($p = 0.0002$). Since we observed increased densities of microglia in two functionally and anatomically disparate cortical areas, we suggest that these immune cells are probably denser throughout cerebral cortex in brains of people with autism.

Keywords Microglia · Autism · Fronto-insular cortex · Visual cortex

Introduction

The brain is substantially isolated from the body's immune system by the blood–brain barrier, which restricts the passage of most immune cell types and proteins from capillaries into brain tissue. The brain has its own immune system based on microglia, which are derived from the macrophage lineage and reside throughout the brain, where they mount defenses against invading microorganisms and clear damaged tissue and metabolic waste (Graeber and Streit 1990, 2010). This is achieved through phagocytosis, in which the microglia ingest these substances.

Nimmerjahn et al. (2005), Davalos et al. (2005) and Wake et al. (2009) directly observed the activity of microglia in intact living mouse brains using two-photon microscopy in animals that express green fluorescent protein specifically in microglia. Their experiments showed that microglial cell bodies are relatively stationary, but their fine processes are in constant motion on a minute-to-minute basis. They observed that the microglial processes continually probe the immediate area, so that the population conducts a complete surveillance coverage of brain tissue every few hours. When the microglial processes encounter damaged tissue, metabolic byproducts such as oxidized lipoproteins, or invading microorganisms, they respond by expanding and engulfing these substances and transporting them back to the microglial cell body where they are stored for an indeterminate period of time. The microglia contact other types of glia and neurons as part of their constant surveillance, but when they encounter other microglia there is mutual repulsion of their processes, which may account for their relatively uniform spacing. There is also evidence that microglial processes can strip synapses away from their dendrites, suggesting that microglia may have another role in modifying neuronal connections in development and plasticity (Blinzinger and Kreutzberg 1968; Graeber et al. 1993; Kreutzberg 1996; Paolicelli et al. 2011). Wake et al. (2009) reported through in vivo imaging that microglia make transient direct contact with synapses, and that the frequency of this contact is

N. A. Tetreault (✉) · A. Y. Hakeem · S. Jiang · B. A. Williams · B. J. Wold · J. M. Allman
Division of Biology, California Institute of Technology, 1200 East California Blvd., MC216-76, Pasadena, CA 91125, USA
e-mail: nicolet@caltech.edu

E. Allman
Comprehensive Autism Center, 2604 B El Camino Real #214, Carlsbad, CA 92009, USA

dependent on neural activity. In experiments in visual cortex, microglia contact frequency was decreased by silencing the visual input by injecting tetrodotoxin into the eyes (Wake et al. 2009). Inducing neural degeneration by transient ischemia increased the duration of microglial contact with synapses which was followed by synapse elimination (Wake et al. 2009).

Microglia are closely related developmentally and functionally to macrophages. Both originate from the monocyte lineage in the bone marrow. Microglia first appear in small numbers in the brain during embryogenesis, but they emerge prominently during the early postnatal period when they enter the brain from the bloodstream to form what has been called the fountain of microglia, in which they migrate along the course of the fibers of the corpus callosum to all parts of the brain (Imamoto and Leblond 1978). The initial population of microglia can be augmented by subsequent invasion into the brain of circulating macrophages, which apparently assume the microglial phenotype after entering neural tissue (Schmid et al. 2009). In a preliminary study of gene expression, we observed in some of our autistic cases increased expression of a network of genes centered on interleukin-6 (Tetreault et al. 2009). Interleukin-6, together with several other genes in the network, is characteristic of activated versus quiescent microglia (Thomas et al. 2006). People with autism have significantly increased cytokines in frontal cortex and elevated levels of cytokines in the cerebrospinal fluid compared to control subjects (Li et al. 2009; Zimmerman et al. 2005) and there is evidence for immune system dysfunction in the development of autistic children (Ashwood et al. 2006; Chez and Guido-Estrada 2010). These observations motivated us to conduct a quantitative study of the density of microglia in brains of individuals with autism compared to controls. Our goal in this work is to quantify microglial differences between subjects with autism and age-matched controls in two cortical areas, fronto-insular cortex (FI) and primary visual cortex (VC). Multiple lines of evidence have previously implicated FI in autism (Allman et al. 2005; Di Martino et al. 2009; Santos et al. 2011); VC was selected because of its functional difference and anatomical distance from FI, in an effort to span the diversity within neocortex.

Methods

Tissue Samples

Formaldehyde-fixed (8 % solution) human right FI and right VC tissue from subjects with autism and controls was obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland-Baltimore, as shown in Table 1. FI was identified based on criteria such as the presence of the Von Economo neurons and sulcal location (Allman et al. 2010) and corresponds to the posterior part of Brodmann's area 47. VC was identified by using the calcarine sulcus as a landmark; the dissections involved the sulcal lip corresponding to Brodmann's areas 17 and 18. The NICHD Brain and Tissue Bank for Developmental Disorders provided detailed clinical records, with personal identification removed, for each individual with autism whose brain we studied, as summarized in the phenotypic descriptions in Table 2. To confirm the diagnosis of autism, the medical records of each person with autism were reviewed in depth by a clinical psychologist (EA) who specializes in autism. In each case we have at least one thorough clinical description of the subject by either a psychologist or psychiatrist. Ten of our eleven subjects with autism had the autistic diagnostic interview-revised (ADI-R), which is the result of a structured interview with a parent of the individual with autism. Three of the individuals with autism had ADIR records, but the actual scores were not in the file. One individual with autism had a childhood autism rating scale and met the criteria for an autism diagnosis. The records additionally include measures of behavioral development such as the Bayley tests, as well as a history of medications and other health issues reported by physicians and clinical psychologists, described in Table 2.

Sectioning and Immunocytochemistry

Samples were sectioned in the coronal plane at 50 μm on a microtome with a vibrating blade (Microm HM 650 V) in 0.1 M phosphate buffer solution (PBS) and stored in well dishes with PBS and sodium azide. The microglia were immunocytochemically stained with an antibody to IBA1 (ionizing calcium adaptor molecule-1), the gene product of the Aif1 gene (allograft inflammatory factor 1), raised against the C-terminus of IBA1, which labels microglia and monocytes. We used the IBA1 antibody because it yields excellent and selective staining of microglia in formaldehyde-fixed human archival brain tissue (Streit et al. 2009). The utility of IBA1 for the study of microglia has also been shown through expression of the IBA1 gene coupled with enhanced green fluorescent protein in experiments employing 2-photon microscopy to image the development and motility of this class of cells in the brains of living mice (Hirasawa et al. 2005; Wake et al. 2009). We used a concentration of 1:1,000 of IBA1 antibody (Wako, Code No. 019-19741). Four batches of immunostaining were performed including duplicate sections from both FI and VC of each of the subjects, and each of the staining procedures showed consistent and robust immunostaining across the sections. Free-floating sections were rinsed with

Table 1 Autistic and neurotypical control subjects used for microglial density measurements

ID	GUID	Age	Sex	Cause of death	Brain weight (g)	PMI (h)	FI	VC
Autistic								
M4021	NDAR_INVUX206VRV	3a	M	Drowning	1,330	15	X	X
M4029	NDAR_INVRX268EH4	3b	M	Drowning	1,130	13	X	X
UMB4671	Not provided	4	F	Fall from 9th story	1,320	13	X	X
UMB797	NDARYX624FEY	9	M	Drowning	1,175	12	X	
M2004	NDAR_INVAK979XTP	10	M	Drowning	1,400	25	X	X
UMB4305	NDARWL137ER1	12	M	Serotonin syndrome	1,360	13	X	
UMB4315	NDAR_INVHD069UM7	14a	M	Natural	1,590	22	X	X
UMB4899	NDAR_INVGW538MM3	14b	M	Drowning	1,450	9	X	X
UMB5278	NDARYH540PL4	15	F	Drowning with seizure	1,417	13	X	X
UMB4999	Not provided	20	M	Cardiac arrhythmia	1,427	14		X
UMB5176[1]	NDARHU383HFF	22	M	Subdural hemorrhage	1,525	18	X	X
Control								
UMB5282	Not provided	2	M	Asphyxia	1,345	16	X	X
UMB1185	Not provided	4a	M	Drowning	1,450	17	X	X
UMB4670	Not provided	4b	M	Commoti cordis	1,470	17	X	X
UMB5387	Not provided	12	M	Drowning	1,750	13	X	X
UMB4925	Not provided	13a	M	Natural	1,650	16	X	
UMB917	Not provided	13b	M	Accident, multiple injuries	1,450	10	X	X
UMB4591	Not provided	16	F	Multiple injuries	1,330	14	X	X
UMB1712	Not provided	20a	M	Gunshot to chest	1,500	8	X	X
UMB4727	Not provided	20b	M	Multiple injuries	Not available	5	X	X
UMB1542	Not provided	22a	M	Multiple injuries	1,510	4	X	X
UMB4542	Not provided	22b	M	Multiple injuries	1,460	8	X	X
UMB1713	Not provided	23	M	Head and neck injuries	1,600	8	X	X

The tissue source is NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. The letters in the age column are for the purpose of differentiating subjects of the same age in the graphs in Figs. 2 and 3 *PMI* post-mortem interval, *X* microglia density measurements were made for this structure

PBS and then incubated with 1 % citrate buffer (Chemicon, cat # 21545) for 30 min at 37 °C for antigen retrieval. Sections were rinsed with PBS, treated to remove endogenous peroxidase activity with 0.75 % hydrogen peroxide and methanol for 20 min, and then rinsed with PBS. The blocking step, to eliminate random antibody binding, used 0.1 % Triton X-100, 4 % normal goat serum (NGS), 1 % BSA, and 3 % dry milk in PBS for 3 h. Primary antibody was incubated for 38 h at 4 °C in a PBS solution that included 0.1 % Triton X-100, 2 % NGS and 1:1000 anti-IBA1. Sections were then rinsed with PBS, incubated with biotinylated anti-rabbit (BA-1000,Vector Laboratories) at 1:200 for 2 h, and then rinsed again with PBS. A Vectastain Elite ABC kit (pk-6100, Vector Laboratories) was used for the avidin–biotin-peroxidase method, then sections were incubated for 30 min. After sections were once again rinsed with PBS, immunoreactivity was visualized by using a chromagen, 3′-diaminobenzidine and nickel (SK-4100, Vector Kit). Null control sections were incubated without primary antibody and incubated with goat IgG at the same concentration as the primary antibody. No immunostaining was observed in these control sections.

Quantification of Microglial Densities

Microglial density in FI and VC was measured blind to phenotype and quantified using the program Stereo Investigator (MBF Bioscience, Williston, VT) with a Reichert Polyvar microscope equipped with a motorized stage and a camera for visualization. All sections were quantified in at least two separate replications with different regions of interest, and some sections were quantified up to four times with both different and identical regions of interest. For all of the samples, duplicate sections of FI and VC were classified and quantified for reproducibility. Independent raters quantified and classified blind random sections to replicate the method. The represented density measure is an average of the blind replicated runs. Quantification was

Table 2 Below is a phenotypic description of the autistic subjects including age, gender, seizure status, medications, medical history, cause of death, PMI

Patient ID	Age and gender	PMI (hours)	Cause of death	Seizure disorder	Medication	Additional medical hist	ADI-R[a]	Neuropath report or autopsy	Patient summary	Respirator or traumatic death state
M4021	3 Years Male	15	Drowning	No	None reported	None reported	Completed not in file A: B: C: D:	None provided	Rigid routine, many repetitive[f] and aggressive behaviors. No gestures for communication, auditory sensitivity	None reported
M4029	3 Years Male	13	Drowning	No	None reported	None reported	Completed not in file A: B: C: D:	None provided	Autistic regression[d] at 24 months, aggressive behaviors, negative response to several sensory stimuli and ran from sounds	Found in a canal and resuscitated with CPR. Five hours on respirator
UMB4671	4 Years Female	13	Fall 9 stories	No	None	Diaper rash	A: 26 B:13 C: 3 D: 5	Autopsy; noted normal brain	Lacked body self awareness and awareness of others[b] (observed by mother); could not identify body parts when tested by a psychologist. Normal hearing. Cognition delayed; no socialization	None reported
UMB797	9 Years Male	22	Drowning	No	Desipramine	ADHD; seizure associated with medication, migraines	A: 24 B: 20 C: 6 D: NA	Neuropath; noted normal brain	No hypersensitivity to sensory stimuli, 8 cortical regions stained with H&E, no microgliosis in cortex, VC had unusually large Meynert cells and an irregular shaped claustrum	Overdose of desipramine a week prior to death; no revival
M2004	10 Years Male	25	Drowning	No	None reported	Hyperactivity	CARS:39 A: B: C: D:	None provided; normal brain noted macro-scopically	Good visual and fine motor skills, ritualistic behaviors and inflexibility, severe language delay	None reported
UMB4305	12 Years Male	13	Serotonin syndrome	Yes	Clonazepam Depakote Olazapine Quetiapine	Pervasive development disorder NOS psychosis NOS ADHD	A: 25 B: 15 C: 8 D: 4	Neuropath; necrosis effects; large macrophage and astrocytes in RH	Very aggressive, destructive and abusive behavior without provocation; special education, lived in group home, unmanageable behavior. Lacked bladder control	Large contusion in right frontal lobe; cystic necrosis
UMB4899	14 Years Male	9	Drowning	Yes	Clonidine Trileptal Zoloft	None reported	A: 22 B: 14 C: 8 D: 4	Neuropath; normal brain and cortex	Loss of verbal skills at 1 year; high levels of sensory interest, compulsions and stereotypy[f] Autistic regression[d]	Found in bottom of pool, CPR revived, on life support for 24 h

Table 2 continued

Patient ID	Age and gender	PMI (hours)	Cause of death	Seizure disorder	Medication	Additional medical hist	ADI-R[a]	Neuropath report or autopsy	Patient summary	Respirator or traumatic death state
UMB4315	14 Years Male	22	Natural	Yes	None reported	None reported	A: 26 B: 16 C: 2 D: 12	Neuropath; brain edema	Prior to death patient had a seizure a week	Brain edema
UMB5278	15 Years Female	13	Drowning	Yes	Depakote Keppra Prozac	None reported	A: 22 B: 11 C: 5 D: 5	None provided; noted normal brain	Noted to have mild autism, high functioning and at grade level, seizures from infancy, second cousin has autism, had spontaneous speech and aggressive behavior	None reported
UMB4999	20 Years Male	14	Cardiac arrhythmia	No	Amoxicillin Depakote Naltrexone Risperdal	Severe mental retardation, aggression, compulsion	Not in file A: B: C: D:	Neuropath; normal brain, increase in leptomengial cells of meninges and gliosis	Self injurious behavior including severe head banging[c], treated with Naltrexone,[e] stereotypy[f], sensitive GI tract and no communication	None reported
UMB5176	22 Years Male	18	Subdural hemorrhage	No	Risperdal	Medical exams reported good health	A: 25 B: 13 C: 7 D: 5	Neuropath; brain intact, deep grey matter structures intact	Nonverbal; makes gestures. Nervousness, behavioral and emotional problems	50 cc left subdural hematoma consistent with head trauma

[a] ADI-R (autism diagnostic interview-revised) test description and cutoffs: qualitative abnormalities in social interaction (A = 10), qualitative abnormalities in communication (B = 7), stereotyped patterns (C = 3), abnormal development (D = 1). All subjects have a psychological evaluation and a Bayley or a developmental test

[b] Minio-Paluello et al. (2009)

[c] Matson and Lovullo (2008)

[d] Goldberg et al. (2003)

[e] Walters et al. (1990)

[f] MacDonald et al. (2007)

performed within a region of interest that spanned layers two to six; microglial distribution appeared relatively homogenous throughout the layers in our samples. Estimated cell counts were performed using the optical dissector probe at 40× magnification (oil immersion NA = 1.0) with a dissector height of 16 μm (flanked by 2.0 μm guard zones), a counting frame of 260 μm × 160 μm and a grid size of 425 μm × 425 μm. To avoid oversampling, we used the Gunderson counting rule such that cells intersecting only 3 of the 6 surfaces of the dissector cube were counted. Microglial density per mm^3 was calculated by dividing the optical fractionator estimate of the number of cells present in the full thickness of the section within the region of interest by the area of the region of interest and the thickness at which the section was cut to account for any tissue shrinkage.

Statistical Analysis

Densities for the subjects with autism and control populations were compared using the Mann–Whitney test with two-tailed p value. Correlation levels between replications were measured using Pearson's r-squared. Possible confounds in the subjects with autism that could alter microglial densities were examined. Binary confounds, including whether death was by drowning and whether seizures were present, were tested using the Mann–Whitney test; a possible confounding correlation with postmortem interval was tested using Pearson's r-squared.

Results

Figure 1 depicts the stereological method and photomicrograph of the microglial quantification method in a brain of an individual with autism. We found significantly higher density in the individuals with autism than in the controls in both FI ($p = 0.0206$, see Fig. 2) and VC ($p = 0.0002$, see Fig. 3). The numbers are represented as the average of the microglial densities for the multiple replications performed in each individual. Comparisons were made using Mann–Whitney tests with two-tailed p values. The repeated quantifications in the same structure are highly significantly correlated: for FI, and VC, $r^2 = 0.6480$, $p < 0.0001$ (Fig. 4) when the blind replications are from the exact ROI the correlation is $r^2 = 0.\ 9780$, $p < 0.0001$ for the intra-rater reliability. Notably, the individuals with autism cluster together in FI and VC, except for a single outlier subject with autism, while the controls all cluster together in both FI and VC.

Figure 2 shows the microglial cell densities in FI of autistic subjects and controls for the combined and averaged data for both microglial quantifications. Individuals

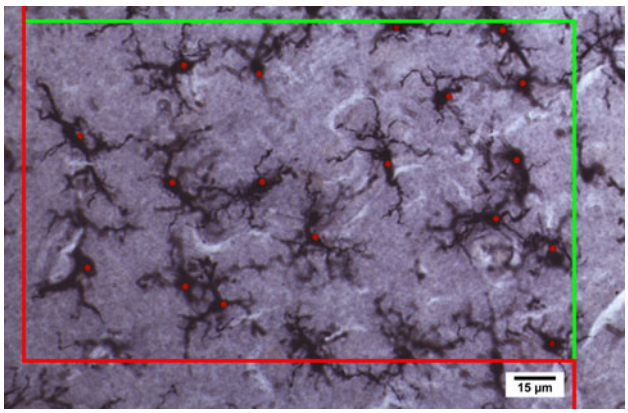

Fig. 1 Stereological procedure for quantifying and identifying microglia in control and the brains of individuals with autism; the *red* and *green* frame defines the borders of the region of interest for counting microglial cells according to the Gundersen et al. (1988) procedure. A microglial cell was included if it was in the counting frame or if the soma crossed the green line and was excluded from the counting when the cell soma crossed the *red line* to avoid oversampling. We used an optical dissector height 16 μm (flanked by 2.0 μm guard zones) and dissector probe at 40× magnification (oil immersion NA = 1.0). Some of the cells are out of focus in the photomicrograph, which is caused by the high numerical aperture of the lens which creates many depth planes through the tissue which is necessary for quantifying cells in three dimensions. Immunocytochemical labeling with Iba1 (1:1000, Wako), a specific marker for microglia and macrophages (Sasaki et al. 2001), in FI of the 14 year old male with autism (UMB4315) (Color figure online)

with autism (n = 10) had significantly higher microglial density ($p = 0.02060$) than control subjects (n = 12) (Mann–Whitney test with two-tailed p value).

The 12-year-old male UMB4305 was a unique case in this group of people with autism because there was no increase in microglial density compared with controls. Although the ADI-R scores for this case are in the autistic range, he was diagnosed as having pervasive developmental disorder not otherwise specified (PDD-NOS), and, in addition, with psychosis NOS, and ADHD. UMB4305 was the only one among all subjects tested who was treated for psychosis, including administration of the drugs quetiapine, olzapine, and risperdal (Table 2). For these reasons, we think this individual may have suffered from a condition distinct from the other individuals who had the autism diagnosis. According to the neuropathology report for UMB4305, "there were three small foci of yellow discoloration noted in the leptomeninges overlying the right antero-inferior frontal pole, right gyrus rectus and left gyrus rectus which measured 0.2 × 0.2 × 0.2 cm. Well-circumscribed regions of shrinkage and slight yellow discoloration were present in the cortical ribbon underlying the discolored leptomeninges. … There was necrosis around the small area of the contusions that included the entire cortical ribbon through layer one. The small frontal lobe contusions had visible macrophages surrounded by

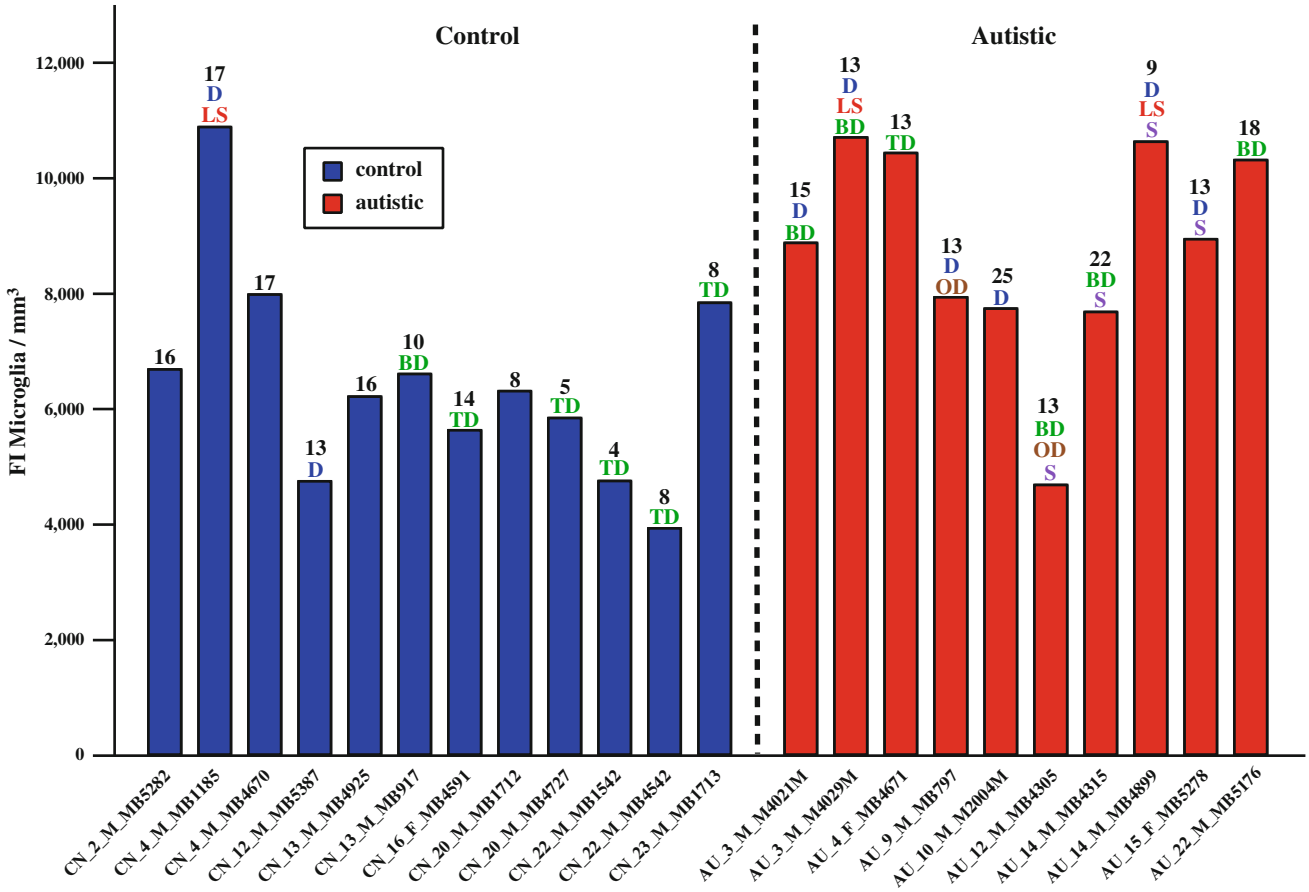

Fig. 2 Microglial densities in FI in subjects with autism and neurotypical brains are represented as the average for the replicated runs. Individuals with autism (n = 10) have a significantly greater density of microglia, the key cellular participants in the inflammatory response in the brain, compared to controls (n = 12) $p = 0.0206$ (Mann–Whitney). LS: known to have spent time on life support. D: cause of death was drowning. BD: brain damage, brain contusion, hemorhage, or edema. TD: traumatic death (MVA, fall) with possible head injury, not explicitly mentioned. OD: drug overdose (not necessarily cause of death). S: seizures (not necessarily cause of death). Numbers in *black* are post-mortem interval in hours

reactive astrocytes observable with a hematoylin-eosin stained sections." The report noted that beyond these local contusions, the cortical layers were normal and the neurons in the cerebral cortex of the fronto-parietal lobe, hippocampus, basal ganglia, and cerebellum were unremarkable.

Figure 3 presents similar data for primary visual cortex (VC). Total microglial densities were significantly greater in VC for the individuals with autism (n = 9) versus the control (n = 11) subjects ($p = 0.0002$ Mann–Whitney test with two-tailed p value). The increase in microglial density is present throughout almost our entire sample of subjects with autism, with ages ranging from 3 years of age to 22. We address the two exceptions to this broad finding, UMB 1185 and UMB 1713, below.

After measuring microglial densities, we consulted Lyck et al. (2009) in which the number of microglia in the cortex of three well-documented neurotypical brains was carefully and comprehensively quantified using a CD45 antibody with unbiased stereology. They reported an average of 3.48 billion CD45 positive cells in the entire human neocortex. Using the estimated value for human neocortical grey volume from Frahm et al. (1982), which is 584,706 mm^3, one can then estimate the density of microglia in the neurotypical human cortex by dividing by the total number of microglia, which is approximately 5,951 (CD45 positive cells) per mm^3 in the total human neocortex (Fig. 5a, b).[1] This is close to our estimated microglial densities for

[1] In Table 5 of Lyck et al. (2009) the column headed "total neocortex" refers to the neocortical gray matter only. In their methods Section 2.2.7, "Estimation of Cell Numbers," they describe their selection of the region of interest, saying, "… followed by delineation the border between white matter and neocortex at 210× magnification (10 × lens) marking the white matter as 'exclusive region'," indicating that their cell number estimates were made from a region that excluded white matter. Further, Fig. 2b from this paper indicates that the brain slices were segmented into "frontal neocortex," "temporal neocortex," "parietal neocortex," "occipital neocortex," and "white matter," implying that the various neocortex segments do *not* include white matter. Thus, in Table 5 the column heads "frontal cortex," "temporal cortex," etc. presumably refer specifically to the gray matter portions of those regions, and "total neocortex" (which is a sum of the other four columns) also includes only gray matter.

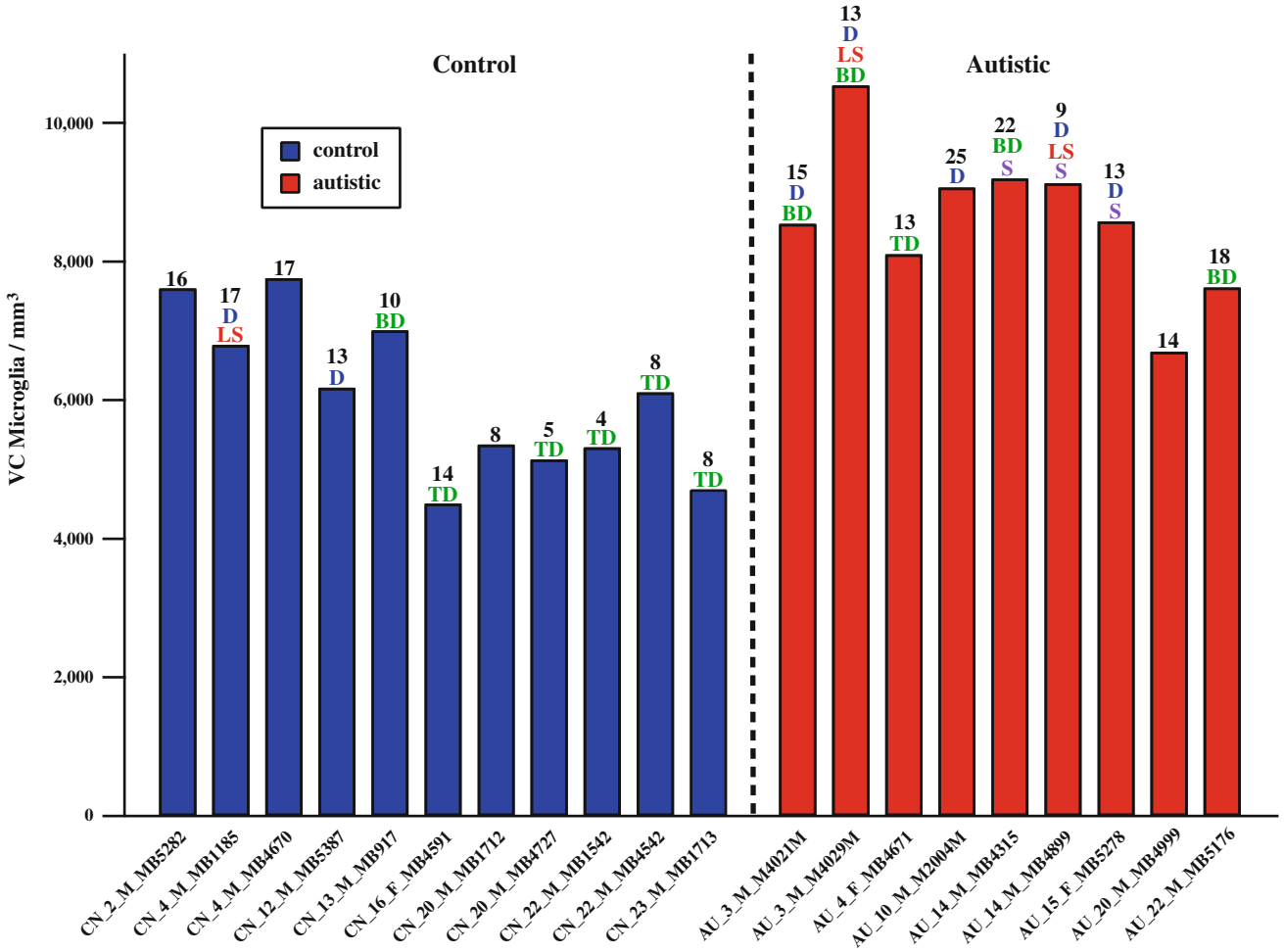

Fig. 3 Microglial densities in visual cortex in autistic and neurotypical brains are represented for the average of the replicated runs. Individuals with autism (n = 9) have a significantly greater density of total microglia, the key cellular participants in the inflammatory response in the brain, compared to controls (n = 11) $p = 0.0002$ (Mann–Whitney)

control FI (6,479 microglia per mm^3) and control VC (6,048 microglial cells per mm^3). In FI, individuals with autism had an 18 % higher microglial density compared to our neurotypical cases, and in VC 21 % higher microglial density compared to our neurotypical cases.

These findings demonstrate that, at the time of death, there were significantly higher microglial densities in the subjects with autism compared to the control subjects, and that this change in microglial density is widespread throughout the cerebral cortex in autism. The microglial densities in FI and VC in the same subject were significantly correlated (both measures were available in 10 controls and 8 autistic subjects for a total of 18 subjects) with Pearson's $r^2 = 0.4285$, $p = 0.0024$ (Fig. 6). This indicates that the elevation in density is consistent between these areas, and probably throughout the cortex, in both subjects with autism and controls.

We tested several confounding variables that could alter microglial densities in FI and VC of the subjects with autism and found no statistically significant relationship between microglial density and drowning versus other causes of death; traumatic versus other causes of death; having been on life support or not; having a recorded drug overdose or not; or having had seizures or not (Table 3). There was no significant difference between the subjects with autism and controls with respect to age of the subjects or post-mortem interval (PMI). However, the controls had significantly greater ($p = 0.0328$) brain weight (1,501 g) versus the subjects with autism (1,374 g) (Mann–Whitney test). This difference was driven mainly by one control subject (M5387) with very high brain weight (1,750 g). This is 310 g greater than the average brain weight (1,440 g) for a 12 year old male (Dekaban 1978), and when the outlier is removed there is no significant difference in brain weight between the subjects with autism and the control subjects. The differences in microglial density between individuals with autism and controls remain significant when the one outlier was removed for density

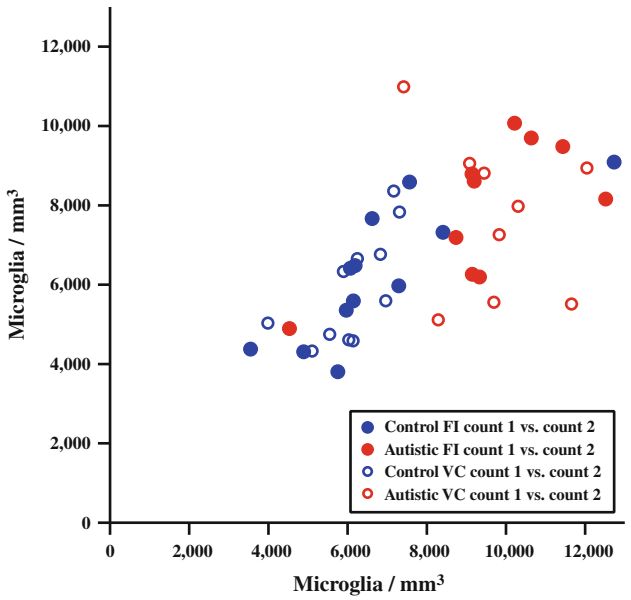

Fig. 4 The repeated blind density measurements are strongly correlated. Density measurement count one in FI versus density measurement count two in FI and density measurement count one in VC versus density measurement count two in VC (Pearson's $r^2 = 0.6480$, $p < 0.0001$) for two different regions of interest (ROI). When the blind replications are from the exact ROI the correlation is $r^2 = 0.9780$, $p < 0.0001$. Notably, the subjects with autism (FI *solid red circles* and VC outlined *red circles*) and controls (FI *solid blue circles* and VC outlined *blue circles*) cluster in FI and VC, except for one autistic outlier in FI (Color figure online)

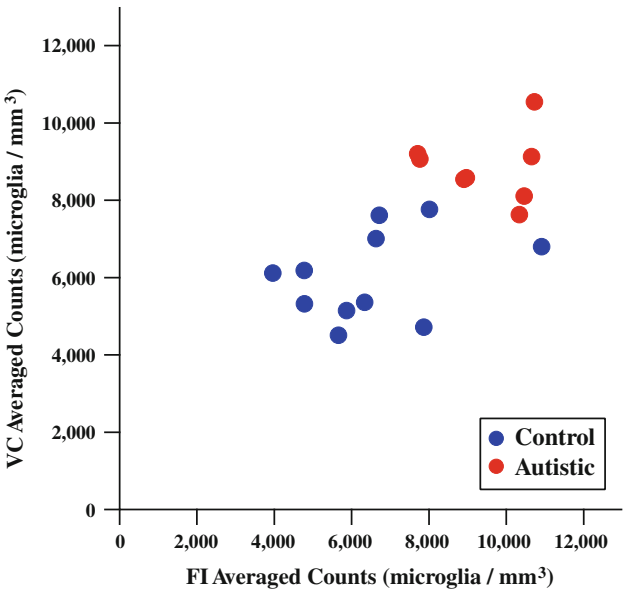

Fig. 6 Microglial densities in FI and VC are significantly correlated. Pearson's $r^2 = 0.4285$, $p = 0.0024$, for the sample of 10 controls and 8 individuals with autism in which measurements were available for both structures. Note that for both structures the individuals with autism (*red*) cluster, as do the controls (*blue*) (Color figure online)

measurements (FI, $p = 0.0257$ and VC, $p = 0.0001$, Mann–Whitney tests). In addition, brain weight and microglial density were not significantly correlated in individuals with autism compared to control cases for FI and VC (Table 3). Morgan et al. (2010) found brain weight was negatively correlated with microglial density in the grey matter, but that the microglial differences between subjects with autism and control subjects persisted when they statistically controlled for brain weight.

Microglial densities were negatively correlated with age in VC in our controls (Pearson's $r^2 = 0.6833$, $p = 0.0017$) and barely missed statistical significance in FI (Table 3). Microglial densities thus tend to decrease with age in controls, while in people with autism the microglial densities remain relatively high and constant with age in both FI and in VC. Finally, microglial densities in VC in controls were negatively correlated with PMI (Pearson's $r^2 = 0.3952$, $p = 0.0383$) but there was no significant correlation in VC for individuals with autism, or in FI for either group (Table 3). Morgan et al. (2010) found that microglial densities were negatively correlated with PMI across their subjects as a whole population but not for controls or people with autism as subgroups.

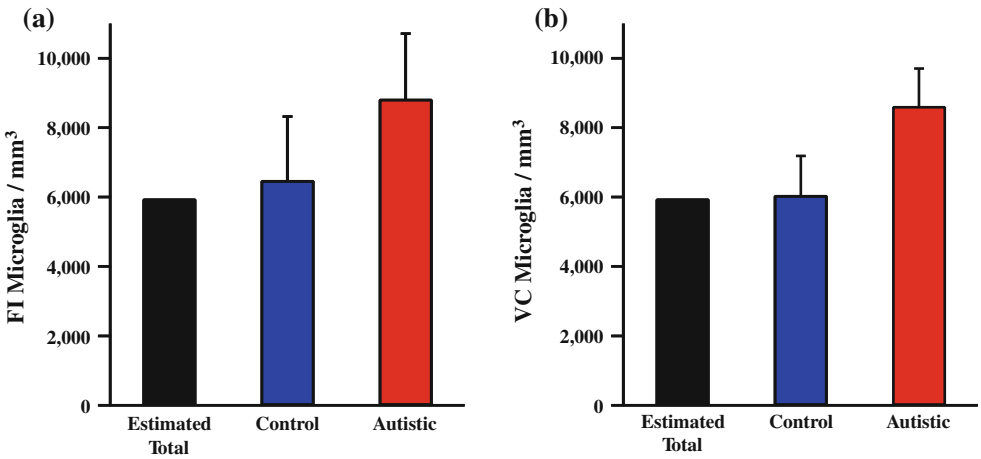

Fig. 5 **a** Average microglial densities for subjects with autism (*red*) and control subjects (*blue*) in FI in comparison to total microglial density (*black*) estimated from data in Frahm et al. (1982) and Lyck et al. (2009). **b** Average microglial densities in VC. *Error bars* represent the standard deviation (Color figure online)

Table 3 Confound statistics for the autistic cases

Confound	FI	VC
Drowning	FI autistics (6 drowning, 5 non-drowning): total density drowning versus other COD, $p = 0.7619$ (ns)	VC autistics (5 drowning, 4 non-drowning): total density drowning versus other COD, $p = 0.2857$ (ns)
Seizures	FI autistics (4 seizures, 6 no seizures): total density seizures versus no seizures, $p = 0.2571$ (ns)	VC autistics (3 seizures, 6 no seizures): total density seizures versus no seizures, $p = 0.7143$ (ns)
PMI	FI autistics, total density versus PMI, N = 10, Pearson's $r^2 = 0.0658$, $p = 0.4743$ (ns)	VC autistics, total density versus PMI, N = 9, Pearson's $r^2 = 0.00159$, $p = 0.9189$ (ns)
	FI controls, total density versus PMI, N = 12, Pearson's $r^2 = 0.2628$, $p = 0.0883$ (ns)	VC controls, total density versus PMI, N = 11, Pearson's $r^2 = 0.3952$, $p = 0.0383$ (significant)
Brian weight	FI autistics, total density versus brain weight, N = 10, Pearson's $r^2 = 0.0077$, $p = 0.8095$ (ns)	VC autistics, total density versus brain weight, N = 9, Pearson's $r^2 = 0.1311$, $p = 0.3384$ (ns)
	FI controls, total density versus brain weight, N = 11, Pearson's $r^2 = 0.00296$, $p = 0.6126$ (ns)	VC controls, total density versus brain weight, N = 10, Pearson's $r^2 = 0.0295$, $p = 0.6348$ (ns)
Age	FI autistics, total density versus age, N = 10, Pearson's $r^2 = 0.0080$, $p = 0.8054$ (ns)	VC autistics, total density versus age, N = 9, Pearson's $r^2 = 0.3477$, $p = 0.0947$ (ns)
	FI controls, total density versus age, N = 12, Pearson's $r^2 = 0.3159$, $p = 0.0572$ (ns)	VC controls, total density versus age, N = 11, Pearson's $r^2 = 0.6833$, $p = 0.0017$ (significant)

Drowning, seizures, PMI age and brain weight do not account for the increase in microglial density for autistics compared to the controls. The controls had significantly greater ($p = 0.0302$) brain weight (1,501.4 g) verses the autistics (1,356.7 g). This difference was driven mainly by one control subject (M5387) with very high brain weight (1,750 g) which is 310 g greater than the average brain weight (1,440 g) for a 12 year old male (Dekaban 1978) and when the outlier is removed there is no significant difference (ns) for brain weight comparing the autistic and control cases. The controls have a significant correlation for microglial density with age in VC ($r^2 = 0.6833$ and $p = 0.0017$), where over time the microglia decrease with age and a similar trend occurs in FI but does not reach statistical significance

We found that FI of two control subjects had unusually high microglial densities compared to the other controls. They were UMB1185, the 4-year-old control case, and the 23-year-old control UMB1713, who had suffered from head and neck injuries. The injuries sustained by UMB1713 are such that could cause an increase in microglial density if death was not immediate (Engel et al. 2000, Loane and Byrnes 2010). Both of these individuals showed increases in microglial densities in FI, but not in VC. By contrast, our subjects with autism had global increases in microglial densities, shown both in FI and in VC. This regional difference suggests the possibility of injury-related pathology in these two controls.

Discussion

We observed increased densities of microglia in two disparate cortical areas. One possibility is that these increased densities reflect abnormalities specific to these particular cortical areas, since there is evidence that each is involved in autism, or alternatively these results may reflect a widespread difference that occurs throughout the cortex or even much of the brain. Consistent with the possibility that the effect is pan-cortical, Morgan et al. (2010) reported an increase in microglia in subjects with autism in dorsal lateral prefrontal cortex (dlPFC) compared to controls, and found an increase in somal size in microglia in subjects with autism in grey and white matter. Five of Morgan et al.'s cases with autism overlap with those used in our study (Table 4). We found that the subjects with autism we had in common with Morgan et al. showed an increase in microglial density in both FI (five subjects in common) and VC (four subjects in common), which is consistent with Morgan et al.'s findings in dlPFC. In addition, Morgan found five of the thirteen individuals with autism had an increase in microglial activation (Morgan et al. 2010). Precedent for Morgan's and our microglial observations comes from Vargas et al. (2005) who found significantly more microglial activation in the cerebellum of autistic brains versus controls and a trend toward more microglial activation in the middle frontal and anterior cingulate cortices, although the cortical results were not statistically significant. One of our individuals with autism was used in the Vargas study (Table 4) (Vargas et al. 2005).

Our methodologies differed, however, in several respects from those of Morgan et al. (2010). We quantified microglia in two cortical regions, FI and VC, consistently in the right hemisphere, whereas Morgan quantified a single region, dlPFC, using either the right or left hemisphere. The reports of increased microglial densities are consistent, but there are differences in density measurements in Morgan's and our studies. The differences in density measurements for the individuals with autism and controls can be attributed to our differing calculations and consideration of the shrinkage factor within the tissue. To account

Table 4 Autistic cases used in the Vargas, Morgan and our study for microglial densities

ID	GUID	Vargas et al. (2005)	Morgan et al. (2010)	Tetreault et al. (this study)
UMB797	NDARYX624FEY	X	X	X
M4021M	NDAR_INVUX206VRV		X	X
M4029M	NDAR_INVRX268EH4		X	X
M2004M	NDAR_INVAK979XTP		X	X
UMB4899	NDAR_INVGW538MM3		X	X
UMB4671	Not provided			X
UMB4305	NDARWL137ER1			X
UMB4315	NDAR_INVHD069UM7			X
UMB5278	NDARYH540PL4			X
UMB4999	Not provided			X
UMB5176	NDARHU383HFF			X

For this study we quantified two regions in cortex, FI and VC which have not previously been quantified and showed that six additional autistic cases have increased microglial density measures. An X indicates that the subject was evaluated in the study

for shrinkage, we calculated the microglial density per mm^3 by dividing the optical fractionator estimate of the number of cells present in the full thickness of the section within the region of interest by the area of the region of interest and the thickness at which the section was cut. Our results for control samples are very close to values calculated for microglia based on the total number of microglia in the entire neurotypical cortex determined through stereology (Lyck et al. 2009) and cortical volumes (Frahm et al. 1982) (see Fig. 5a, b).

The differences between our study and Vargas et al. (2005) are that they stained microglia with an antibody to HLA-DR and used an area fraction quantification method based on the Delesse sampling procedure (Gundersen et al. 1988). That method gives an estimate of the fractional area of the region of interest covered by the cell type being measured. The Delesse method does not, however, produce cell numbers or three-dimensional densities. By contrast, we stained with an antibody to IBA1 and measured microglial density in our tissue. Though the specifics of antibody and methods differed, our data taken together with Vargas et al. (2005) and Morgan et al. (2010) point to elevated microglial density in autism, possibly involving the entire cerebral and cerebellar cortices. This argues that further investigation of microglial abnormalities and the microglial pathways in people with autism may be important for understanding the cellular basis of the autism phenotype.

There are also some caveats. We cannot be sure that IBA1 stains all microglia, and there is evidence for microglial heterogeneity (Carson et al. 2007; Mittelbronn et al. 2001; Schmid et al. 2009). However, the spacing of the stained microglia we have observed is consistent with complete coverage of a relatively regular array of microglia in the cortex. In addition, we found microglial densities in control FI and VC that are near the expected densities calculated from Lyck et al. (2009) and Frahm et al. (1982). On average the individuals with autism had 18–21 % higher microglial density in FI and VC compared to neurotypical subjects. How and when does the increased density of autistic microglial arrays arise, and how is it maintained? Of course we have no data prior to the time of death, but the consistency of results among 10 subjects with autism of differing ages argues that people with autism have developed a remarkably stable steady-state microglial density. Given the age range, this is probably established before age three. It is not clear how long the increase in microglia persisted in each of the subjects with autism, but our results show that control subjects have a significant negative correlation between microglial density in VC and age, indicating that microglial densities normally decrease throughout childhood and early adulthood in neurotypical subjects. However, in people with autism, there is a relatively steady condition of increased microglial density from childhood into adulthood. It seems possible that some persistent stimulus is the cause of this sustained higher level of microglial density in the subjects with autism. Imaging experiments of quiescent microglia in intact living cortex suggest that they conduct a complete surveillance of the cortex every few hours (Davalos et al. 2005; Nimmerjahn et al. 2005). The greater density, and thus closer spacing of the microglia, in brains of individuals with autism compared with control brains, suggests that this surveillance is more intense in autism.

Sickness behavior results from systemic infection and/or inflammation, driving an increase in signals to the brain that cause changes in metabolism, social withdrawal, appetite suppression and a general ill feeling (Exton 1997; Hart 1998; Perry 2010). Sickness behavior is another example of how a systemic infection or its related

inflammation can alter both behavior and the inflammatory response in the brain. There is evidence that maternal viral infection in the first trimester and bacterial infection in the second trimester are correlated with an increase in offspring reported to have autism (Atladóttir et al. 2010). In a recent microarray analysis of gene expression in brains of individuals with autism compared to controls, Voineagu et al. (2011) found a module of enriched immune and microglial genes, although these genes have not been found in genome wide association studies that have sought to identify genes that predispose to autism. Voineagu et al. (2011) conclude that the enriched gene expression of immune and microglial genes observed in their study has a non-genetic etiology and may reflect internal or external environmental influences, which suggests the possibility that the sustained higher levels of microglia density in people with autism may also be environmentally mediated.

Chez and Guido-Estrada (2010) report that a subset of people with autism have a consistent pro-inflammatory condition of the brain and cerebral spinal fluid and proposed that a systemic infection of the mother may lead to inflammation in the brain and autism. A recent report from Wei et al. (2011) found an increase of IL6 in cerebellar cortex in subjects with autism, which could alter cell migration and disrupt imperative circuits for normal development (Wei et al. 2011). In a mouse model of maternal infection for offspring brain development, it has been reported that offspring from a mother having a single injection of IL6 during pregnancy alters fetal brain development (Smith et al. 2007) which indicates that a maternal infection can impact brain development and may play a critical role in autism. Girard et al. (2010), using a lipopolysaccharide (LPS) mouse model of maternal inflammation, found that a single treatment of an IL-1 receptor antagonist, concurrent with the LPS injection, had the result that the IL-1 receptor antagonist protected against maternal placental inflammation and the offspring had normal brain development. Furthermore, it is well documented that peripheral infection can dysregulate inflammation in the brain and increase monocyte infiltration into the cerebral cortex (D'Mello et al. 2009); it is also reported that people with autism have elevated levels of cytokines (Chez and Guido-Estrada 2010) which may disrupt the homeostatic balance in the cortex resulting in a greater density of microglia.

Are Microglia Predators or Protectors?

The increased microglial densities we observed in the cortices of our subjects with autism appear to be a robust discriminator between the brains of people with autism versus neurotypical brains, and these findings raise a major question. Are markedly increased numbers of microglia among the originators of the pathologic processes in autism, or are they a response (perhaps even a protective one) to some other aspects of this condition? Microglia have neuroprotective functions including the phagocytosis of invading microorganisms and metabolic waste. The increase of microglial densities in individuals with autism could be a function of neuroprotection in response to harmful microorganisms.

In contrast, microglia can also phagocytize synapses and whole neurons, thus disrupting neural circuits. For example, when the axons of motor neurons are cut, the microglia strip them of their synapses (Blinzinger and Kreutzberg 1968; Cullheim and Thams 2007; Graeber et al. 1993). Another example of the disruption of circuitry arises from the direct phagocytosis of neurons. Neurons communicate with microglia by emitting fractalkine, which appears to inhibit their phagocytosis by microglia. Deleting the gene for the microglial fractalkine receptor (Cx3cr1) in a mouse model of Alzheimer's disease has the effect of preventing the microglial destruction and phagocytosis of layer 3 neurons that was observed in these mice in vivo with 2-photon microscopy (Furhmann et al. 2010). In particular, Cx3cr1 knockout mice have greater numbers of dendritic spines in CA1 neurons, have decreased frequency sEPSCs and had seizure patterns which indicate that deficient fractalkine signaling reduces microglia-mediated synaptic pruning, leading to abnormal brain development, immature connectivity, and a delay in brain circuitry in the hippocampus (Paolicelli et al. 2011). In summary, the increased density of microglia in people with autism could be protective against other aspects of this condition, and that a possible side-effect of this protective response might involve alterations in neuronal circuitry.

Microglial Defects as Causes of Disease

By contrast, there are diseases that arise from intrinsic defects in the microglia themselves which can cause stereotypic behavioral dysfunctions. A naturally occurring genetic defect in human microglia is the cause of a remarkable neuropsychiatric disease that was first observed in Japan and Finland, but has subsequently been found throughout the world. Nasu-Hakola disease is caused by a defect in the gene TREM2 or DAP12 which together form a receptor complex which is strongly expressed in microglia but not in astroglia or oligodendroglia (Paloneva et al. 2002; Sessa et al. 2004). In the Allen Brain Atlas, DAP12 is preferentially expressed in olfactory, anterior cingulate, and insular cortices in the mouse (http://mouse.brain-map.org/experiment/show/70523695). These defects in TREM2 or DAP12 impair the capacity of the microglia to phagocytose damaged tissue and increase the secretion of inflammatory cytokines in the olfactory, insular and

cingulate cortices resulting in microglia-mediated dementia specific to these structures (Bianchin et al. 2004; Neumann and Takahashi 2007). Bianchin et al. (2004) report that at around age 35 in affected patients there are: "[i]ncipient personality changes that can only be noticed by relatives and close friends. The behavioral alterations then become progressively more evident during the next months. The patients start to present silly and facetious behavior, lack of insight, social inhibition, and other unrestrained behavior. Sometimes they seem to have a euphoric attitude and are easily distractible, seemingly lacking adequate associated emotional components. As the disease progresses, the patients evolve to a state of profound dementia." The remarkable behavioral specificity of the microglial defect in Nasu-Hakola disease shows that the microglia can influence social behavior in a highly specific manner.

Another stereotypic behavioral defect arising from abnormal microglia is obsessive grooming in mice with a mutation of the gene Hoxb8 (Chen et al. 2010). Hoxb8 is expressed only in the microglia in the adult mouse brain, and these cells originate in spinal bone marrow (Chen et al. 2010). When adult mice with the Hoxb8 mutation were irradiated so as to kill the bone marrow and then received bone marrow transplants with the intact gene, the mice recovered from their excessive grooming pathology, their skin lesions healed, and their fur grew back to normal. When normal mice were irradiated and received bone marrow from donors with the mutated Hoxb8 gene, they developed the excessive grooming pathology. With these experiments and a variety of other elegantly executed controls, Chen et al. (2010) demonstrated that the Hoxb8 mutation with expression restricted to the microglia caused the pathological grooming behavior. This behavior resembles obsessive–compulsive disorder in humans, which involves abnormalities in orbito-frontal and anterior cingulate cortices (Graybiel and Rauch 2000). These structures are also implicated in autism (Allman et al. 2005; Di Martino et al. 2009; Santos et al. 2011; Simms et al. 2009). The excessive grooming in the Hoxb8 mice is also reminiscent of the stereotypical behaviors that are commonly found in a subset of individuals diagnosed with autism (Goldman et al. 2009).

Together with the striking changes in social behavior present in Nasu-Hakola disease, these data suggest that the circuitry of anterior cingulate and orbito-frontal cortices may be particularly sensitive to the disruptive effects of abnormal microglia. A strong association between reduced activity in the anterior cingulate and anterior insular cortices (adjacent to orbito-frontal cortex) in social tasks in subjects with autism versus controls was revealed in a meta-analysis of 24 functional imaging studies (Di Martino et al. 2009), and the reduced activity in these structures in autism may also be related to the apparent vulnerability of these structures to microglial disruption. This vulnerability might also be related to the preferential expression DAP12 in the anterior cingulate and insular cortices. Area FI investigated in our study corresponds to the ventral part of anterior insular cortex. Thus, while changes in microglial density appear to be widespread in brains of autistic individuals, some areas may be more vulnerable than others to its effects.

Visual Abnormalities in Autism

When we began this investigation we anticipated microglial abnormalities in the frontal cortices because many lines of evidence suggest that these structures are involved in autism (Allman et al. 2005; Courchesne and Pierce 2005; Di Martino et al. 2009). We included visual cortex based first on its lack of involvement in prominent social and homeostatic functions and its physical distance from FI. Yet, abnormalities in visual behavior are among the first signs of autism in infancy. Beginning at the end of the first year, the earliest signs of autism include atypical eye contact and visual tracking, and prolonged fixation, a tendency to perseverate visual attention on an original stimulus when presented with a competing stimulus (Zwaigenbaum et al. 2005).

The increased microglial densities in visual cortex may be representative of a pan-cortical microglial phenotype related to the autistic phenotype associated with perceptual integration. In Kanner's original description of autism he emphasized his patients' intense fixation on detail and "inability to experience wholes without full attention to the constituent parts" as a characteristic feature of the disorder (Kanner 1968). Frith (2004) drew attention to the tendency for typically developing children and adults to process information for meaning and gestalt (global) form, often at the expense of attention to or memory for details and surface structure. Happe and Frith (2006) proposed that autistic subjects show "weak central coherence," a processing bias favoring local information, and a relative failure to extract the gist or "see the big picture" in everyday life. The tendency of individuals with autism to focus on detail at the expense of global perceptions has been experimentally verified in many studies and may account in part for impairments in the recognition of faces (Behrmann et al. 2006; Happe and Frith 2006).

This difficulty perceiving the gist or global features of a stimulus configuration by subjects with autism may be analogous to the difficulties experienced by subjects with autism in making rapid intuitive decisions (Allman et al. 2005). A variant of the "weak coherence" theory applied to frontal lobe function and specifically linked to activated microglia and their possible role in altering the development of this structure was proposed by Courchesne and Pierce (2005). Happe and Frith (2006) propose that "weak

coherence" in autism is due to reduced connectivity throughout the brain due to lack of synchronization of neural activity (Brock et al. 2002) or lack of connecting fibers (Just et al. 2004) or lack of top-down connections (Frith 2004). A similar disruption of global connectivity might disrupt the integrative functions of FI in social behaviors as suggested by the reduced activity in this area in subjects with autism versus controls (Allman et al. 2010; Di Martino et al. 2009). An increased density of microglia throughout the cortex in people with autism might contribute to these phenomena through alterations in the neuronal circuitry.

Summary

We found significantly increased microglial densities in individuals with autism in two widely separated and functionally different cortical areas, FI and VC. In light of our findings, as well as increased densities in the cerebellum (Vargas et al., 2005) and dorsal lateral prefrontal cortex (Morgan et al. 2010), we propose that microglial densities are elevated throughout the cerebral and cerebellar cortices in individuals with autism. Future studies are needed to explain the mechanisms responsible for the increased densities and the relationship between this phenomenon and the behavioral manifestations of autism.

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