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Developmental microglial priming in postmortem autism spectrum disorder temporal cortex

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ABSTRACT

Microglia can shift into different complex morphologies depending on the microenvironment of the central nervous system (CNS). The distinct morphologies correlate with specific functions and can indicate the pathophysiological state of the CNS. Previous postmortem studies of autism spectrum disorder (ASD) showed neuroinflammation in ASD indicated by increased microglial density. These changes in the microglia density can be accompanied by changes in microglia phenotype but the individual contribution of different microglia phenotypes to the pathophysiology of ASD remains unclear. Here, we used an unbiased stereological approach to quantify six structurally and functionally distinct microglia phenotypes in postmortem human temporal cortex, which were immuno-stained with Iba1. The total density of all microglia phenotypes did not differ between ASD donors and typically developing individual donors. However, there was a significant decrease in ramified microglia in both gray matter and white matter of ASD, and a significant increase in primed microglia in gray matter of ASD compared to typically developing individuals. This increase in primed microglia showed a positive correlation with donor age in both gray matter and white of ASD, but not in typically developing individuals. Our results provide evidence of a shift in microglial phenotype that may indicate impaired synaptic plasticity and a chronic vulnerability to exaggerated immune responses.

1. Introduction

Autism spectrum disorders (ASD) are neurodevelopmental disorders characterized by abnormal social communication and restricted/repetitive behaviors (DSM-5; American Psychiatric Association, 2013). The etiology of ASD remains unclear, but emerging evidence in humans and animal models suggest that disruption in the immune system may underlie abnormal brain development associated with cognitive and social deficits in ASD (reviewed in Gottfried et al., 2015; Petrelli et al., 2016). While the central nervous system (CNS) has the capacity to maintain a homeostatic environment for optimal brain function, disturbances caused by external pathogens can activate its intrinsic immune system. Microglia, specialized tissue macrophages residing in the CNS, play a critical role when the immune system is activated. However, while the primary function of microglia was thought to be only phagocytic removal of debris and active tissue scanning (Neumann et al., 2009), recent investigations suggest that these cells function beyond acting as the brain's immune system (reviewed in Wake et al., 2013; Rohan Walker and Yirmiya, 2016). Microglia are involved in synaptic organization (Paolicelli et al., 2011; Tremblay et al., 2011; Schafer et al., 2012; Kim et al., 2016), control of neuronal excitability (Coull et al., 2005), learning and memory (Morris et al., 2013), and brain protection and repair (see figure in Takano, 2015), all of which are critical for development and maintenance of the CNS throughout the lifespan.

Microglia can take diverse morphologies that correlate with their physiological functions. Structurally distinct phenotypes including ramified, intermediate forms, rod and amoeboid microglia were identified in the early 1930s (del Río-Hortega, 1932 and Kershma, 1939). Further examination lead to additional phenotypes including primed, reactive, and dystrophic microglia (Streit et al., 2004; Streit et al., 2009; Taylor et al., 2014; Torres-Platas et al., 2014a,b). Ramified microglia (or "resting" microglia) are involved in surveying the microenvironment of the CNS. Surveillance of the CNS involves monitoring synapses, the

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extracellular milieu and processing of sensory information and indications of stress (Dwyer and Ross, 2016). Other forms, such as intermediate forms, reactive, rod, and dystrophic microglia are indicative of a pathological state of the CNS. For example, rod microglia are observed in animal models of traumatic brain injury (Taylor et al., 2014; Ziebell et al., 2012) and dystrophic microglia are indicative of the senescent state of the CNS, often associated with aging and Alzheimer's disease (Streit et al., 2004, 2009). In other neuropsychiatric disorders (Frick et al., 2013) and degenerative neurological diseases (McGeer et al., 1993; Tischer et al., 2016), aberrant microglial morphology as well as abnormal distribution have been observed. Together this provides evidence that the pathological state of the CNS is reflected in the structural and distributional shift of microglia.

Much like other neuropsychiatric disorders, microglial pathology has been observed in postmortem ASD brains. Vargas et al. (2005) first showed an increase in CSF level cytokines and microglial activation in the cerebellum and medial frontal gyrus in ASD. Following studies used stereological estimations of microglia and showed increased somal volume and density in the dorsolateral prefrontal cortex (dlPFC) white matter (Morgan et al., 2010), visual cortex and frontoinsular cortex (Tetreault et al., 2012). On the other hand, density of microglia did not differ between typically developing individuals and ASD in the amygdala, but abnormal microglial morphology was observed in ASD (Morgan et al., 2014). These studies overall suggest that neuroinflammation is present in ASD, yet demonstrate a spectrum of microglial pathology reflecting the disorder's heterogeneity.

Neuroinflammation can be characterized by either change in microglial density or phenotypic shifts in microglial morphology leading to release of inflammatory cytokines. It may be the case that during development in ASD, increased number of microglia were created (due to genetic predisposition, infection, local brain damage, etc.) and this is reflected in the overall increase in microglial density. However, it is possible that this overall increase in microglial density is driven by a selective increase in a few microglial phenotypes. Also, given the heterogeneity in microglial pathology in ASD, it is also possible that there is a shift in the morphology, due to changes in the microenvironment during development, without an overall microglial density change. Microglia have a critical role during development and their morphology and distribution play a significant role in the process (Harry and Kraft, 2012), but the separate contribution of different phenotypes to the pathophysiology in neuropsychiatric disorders such as ASD and neurological diseases remains almost entirely unknown.

To address this gap, we used an unbiased stereological approach to quantify six morphologically distinct microglial phenotypes (ramified, primed, reactive, perivascular, rod and dystrophic) in postmortem human temporal cortex (including the superior temporal gyrus and fusiform gyrus), which were immunolabeled with an antibody against ionized calcium binding adapter molecule 1 (Iba1). Here, we report human postmortem cortex measures on six distinct phenotypes including ramified, primed, reactive, perivascular, rod and dystrophic. The total density of all microglia phenotypes did not differ between ASD and typically developing individuals. However, there was a significant decrease in ramified microglia in both gray matter and white matter of ASD, and a significant increase in primed microglia in gray matter of ASD compared to typically developing individuals. This increase in primed microglia showed a positive correlation with age in both gray matter and white of ASD, but not in typically developing individuals. In addition, for the first time, we report clusters of microglia present in both white matter and gray matter, which were more frequently observed in ASD compared to typically developing individuals. We discuss these findings in terms of the functional significance of differing microglial morphologies.

2. Materials and methods

2.1. Brain samples and processing

The postmortem brains were obtained from the Brain Bank for Disabilities and Aging in Staten Island, the Autism Tissue Program in Princeton New Jersey, and the NICHD Brain and Tissue Bank for Developmental Disorders at University of Maryland, Baltimore. The brains were hemisected and the right half was fixed with 10% formalin for at least 30 days. Large hemispheric sections were embedded with propylene glycol and serial 50-μm thick sections were cut on a sliding microtome at room temperature. All brains were examined for white matter and vascular disease changes and for evidence of infraction by general pathological procedures such as brain cutting, sampling, and staining. Sections were stored in 50% ethanol and dissected to isolate the temporal cortex (including mainly superior temporal gyrus and partly fusiform gyrus). All ASD donors were diagnosed with the Autism Diagnostic Interview™, Revised (ADI™-R). The demographic summaries of autopsy reports were obtained from the autism brain net website (www.ATPPortal.org) and are shown in Table 1 for autism spectrum disorder (ASD) and typically developing individual (control) donors. Samples for the ASD and typically developing individual donors were age matched, where the mean age of ASD donors was 14.6 years (range 2.8–29) and typical developing individual donors was 14.9 years (range 1.8–32). The mean postmortem interval (PMI) was 14.76 h for ASD and 18.33 h for typically developing individuals. The drug and seizure histories of the ASD donors were obtained from the case documents (ADI™-R and medical records) on the autism brain net website and are shown in Table 2.

2.2. Iba1 immunohistochemistry

Every one section from one 50-um series for each subject was selected for use in the current immunohistochemistry study as described below. Free-floating sections were washed in tap water for five minutes before exposure to 3% H_2O_2 for 30 min at room temperature. The sections were transferred to 0.1 M phosphate buffer solution (PB) and washed thoroughly six times (two minutes per wash on a medium–high speed agitator), then agitated (medium–high speed) in 2.5% normal horse blocking serum for 30 min at room temperature. The sections were transferred to 1:500 diluted Iba1 primary antibody (WAKO Cat# 019-19741, RRID:AB_839504) and agitated at 5 °C for 48 h. Sections were transferred to PB for one wash and treated with anti-rabbit secondary antibody (ImmPRESS HRP Polymer Detection Kit, Vector Labs, Burlingame, CA, USA; MP-7401) for 30 min before additional eight washes with PB (two minutes per wash on a medium-high speed agitator). Iba1-positive cells were visualized with 3,3-diaminobenzidine (DAB) (ImmPACT DAB peroxidase substrate kit, Vector Labs, Burlingame, CA, USA; SK-4105) and mounted onto gelatin-coated slides, dried and coverslipped after dehydration/defatting (50% EtOH-70%-95%-95%-100%-100%-xylene-xylene).

2.3. Microscopy and image acquisition

Virtual sections were created using a digitizing scanning microscope (Axio Scan.Z1, Zeiss, Germany) using 20× objective and acquiring 21 focal planes through the z-axis of each section. Virtual sections were processed with ZEN 2 (Zeiss, Germany) to capture images at 20× objective. Images from gray matter and white matter in each subject were captured and saved as tagged-image file format (TIFF). For area fraction analysis and composites, Helicon Focus 2012 (www.heliconsoft.com) was used to compress the stack images to one flattened image.

Table 1
Summary of autopsy and medical histories of the postmortem tissue used in this study (from the ATP website, www.ATPPortal.org).

Case No.	Diagnosis	Age (years)	Sex	PMI (hrs)	Brain (g)	Age at diagnosis (months)	Cause of death
B-6399	ASD	2.8	M	4	1325	n/a	DR
HSB-4640	ASD	8.5	M	13.8	1740	18	Seiz, HF, RD
AN01293	ASD	9	M	3.75	1690	18	CF
Cal-105	ASD	11.9	M	11	1294	18	DR
UMB-4305	ASD	12.9	M	13	1360	Data in process	SS
UMB-4315	ASD	14.1	M	22	1590	Data in process	Seiz
UMB-4899	ASD	14.4	M	9	1450	12	DR
UMB-4999	ASD	20.8	M	14	1427	24	CA
IBR-93-01	ASD	23	M	14	1610	26	DR
B-6994	ASD	29	M	43	1580	n/a	Seiz
Mean		*14.6*		*14.76*	*1506.6*	*19.33*	
BTB-3958	Control	1.8	M	24	n/a	No diagnosis	n/a
BTB-4235	Control	2.1	F	14	997	No diagnosis	n/a
UMB-1706	Control	8.6	F	20	1340	No diagnosis	CF
UMB-1670	Control	13.3	M	5	1420	No diagnosis	RD
UMB-1790	Control	13.7	M	18	Data in process	No diagnosis	MI
UMB-1322	Control	16.6	M	25	Data in process	No diagnosis	HI
UMB-4590	Control	20.5	M	19	Data in process	No diagnosis	CF
BTB-3960	Control	25.6	F	26	1520	No diagnosis	n/a
IBR-291-00	Control	32	M	14	1540	No diagnosis	HF
Mean		*14.9*		*18.33*	*1363.4*	*n/a*	

PMI = postmortem interval in hours (hrs), Cause of death: CA, cardiac arrhythmia; CF, cardiac failure; DR, drowning; HF, high fever; HI, head injury; MI, multiple injuries; RD, respiratory distress; Seiz, seizures; SS, serotonin syndrome.

Table 2
Summary of autistic donor symptoms and drug history from ATP website (www.ATPPortal.org).

Case No.	Age	Symptoms	Drug History	Seizures
UMB-4305	12.9	Bipolar, Aggression, fecal smearing	Depakote*, Clonazepam*, Seroquel**, Zyprexa**	Yes
UMB-4315	14.1	Restricted, repetitive and stereotyped behaviors	Subtherapeutic medication level	Yes (died)
UMB-4899	14.4	Stereotypy	Subtherapeutic medication level	Yes
UMB-4999	20.8	Aggression, OCD, anxious, restless, biting, head-banging, sleep disorder, gastric distress	Risperdal**, Zoloft***, Prozac***, Naltrexone	No
IBR 93-01	23	Aggression, hyperactivity, sleep disorder	Seroquel**, thioridazine**, propranolol	Yes (died)

The symptoms are obtained from both the ADI-R and medical records of the patient provided on the portal. Drug history is available from the medical records available online.
* Anti-seizure medication;
** Antipsychotic medication;
*** Antidepressant medication.

2.4. Quantitative assessment of Iba1-immunoreactive (Iba1-ir) microglia

Area fraction of Iba1-ir microglia was calculated using image analysis software ImageJ (NIH). For each subject and location (gray matter and white matter), average of 30 images were taken randomly throughout the entire tissue at 20× objective. Images (Fig. 1A) were processed as follows: 8-bit conversion, background subtraction (rolling ball algorithm), gamma correction, normalization, and enhance contrast. The default threshold value was applied to the preprocessed images to measure area fraction (Fig. 1B). Area fraction from each subject was calculated by averaging all the images analyzed for each subject.

Iba1-ir microglial phenotypes in both gray matter and white matter were identified with the criteria discussed in previous reports (for gray matter see Torres-Platas et al., 2014a; Streit et al., 2014; for white matter see Torres-Platas et al., 2014b; for more see Boche et al., 2013). Apart from the traditionally discussed microglia morphology (ramified, primed, reactive and perivascular), two additional microglia morphologies including rod microglia (Taylor et al., 2014) and dystrophic microglia (Streit et al., 2004, 2009) were identified. Density of each phenotype was computed using an unbiased stereological approach with an optical fractionator probe allowing for 3D quantification with a Carl Zeiss Axio Imager M2 (Carl Zeiss, Jena, Germany) connected to a stereology workstation, NewCast software (VISIOPharm 4.3.2, Hørsholm, Denmark). The method estimated the total number of cells per unit tissue volume with an optical probe providing counts through the *z*-axis. The sample process was performed by adding a sampling grid (1434.85 μm × 1434.85 μm) over gray matter and white matter of Iba1-immunolabeled section. Within the grid, the software randomly places a counting frame (207.10 μm × 165.68 μm) in which cells were recognized and counted at 63× objective with immersion oil. Cells within the counting frame were counted and cells outside were not included. Top and bottom guard zones were set at 5 μm of the section thickness. Using the cell number estimation equation (West et al., 1991), each Iba1-ir microglial phenotype was estimated. The density was calculated by dividing the total number of cells per phenotype (*N*) by the calculated total sampling volume (mm^3). All morphological profiling of microglia was carried out by two raters blind to the diagnosis group.

2.5. Statistical analyses

SPSS 22 (SPSS Inc., Chicago, USA) was used for statistical analyses and GraphPad Prism 6 (GraphPad software, San Diego, CA, USA) was used for making graphs. The density for all cell types were compared between ASD and typically developing individuals using an ANCOVA controlling for postmortem interval (PMI) and age. The relationship between microglial density and age was shown with linear regression. The Pearson correlation and non-parametric rank correlations were used. All correlations showed the same trend, but to minimize the effects of outliers and small number of subjects, we report the Kendall's Tau correlation coefficient.

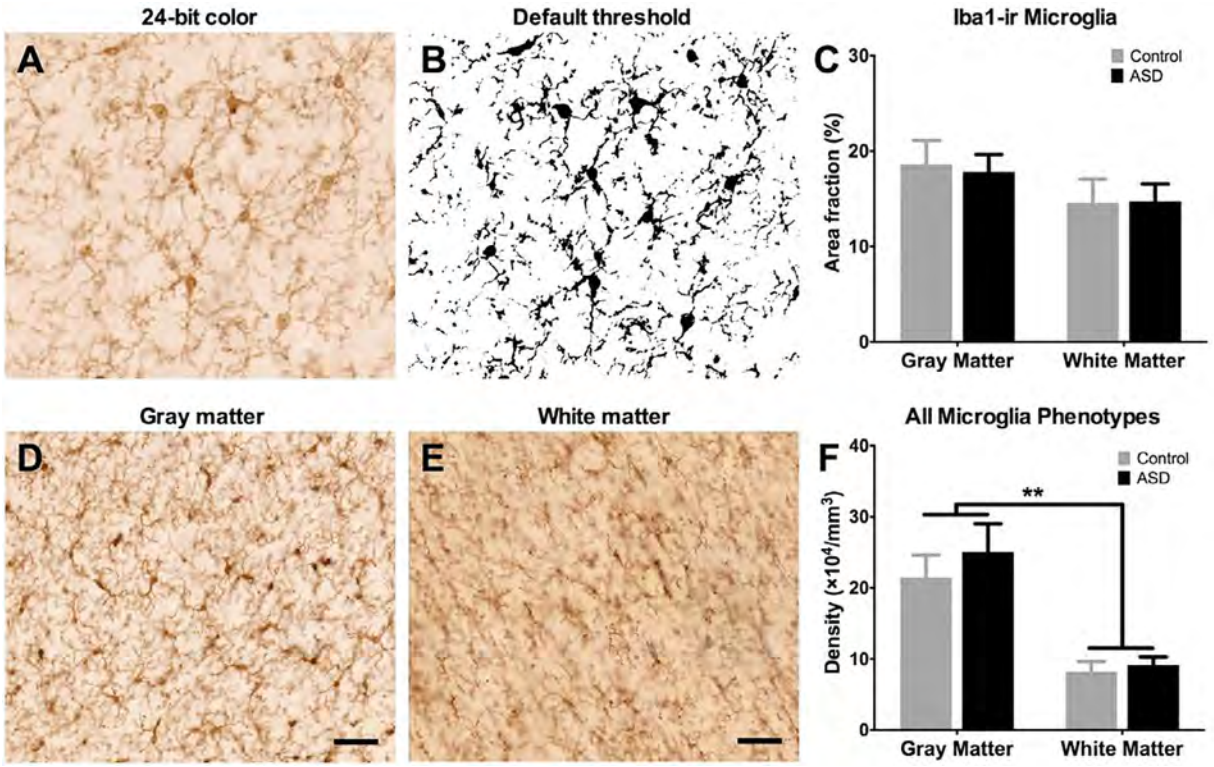

Fig. 1. Overall distribution of Iba1-immunoreactive microglia population in superior temporal gyrus gray matter and white matter. (A) 24-bit color image of a sample 20× objective micrograph. (B) After preprocessing of the 24-bit image, default threshold was applied. (C) Area fraction of Iba1-ir microglia showed no significant difference between the two groups. Micrographs for (D) and (E) were taken from a 12.9-year-old individual diagnosed with ASD at 5× objective. (D) Microglia in gray matter appear evenly distributed across the cortical layer, while (E) in white matter they are aligned to myelinated tracts. (F) Density of all microglia phenotype combined showed no significant difference between the two groups in both gray matter and white matter. There was a significant difference between gray matter and white matter in both groups. Scale bar = 50 μm. $^{**}p < 0.01$

3. Results

3.1. No global difference in Iba1-ir microglia

Before a more rigorous analysis of Iba1-ir microglial morphology, we first examined the area fraction of Iba1-ir microglia to see if there was a global change in Iba1 expression. Area fraction of Iba1-ir in both gray matter ($F(1,15) = 0.07$, $p = 0.803$) and white matter ($F(1,15) = 0.04$, $p = 0.852$) showed no significant difference between the two groups (Fig. 1C). We then examined microglial distribution in gray matter and white matter. Due to its neuroanatomical organization, the distribution of microglia in gray matter (more evenly spread out cortical layers) and white matter (aligned with myelin fibers) was different (Fig. 1D and E). The total densities of all Iba1-ir microglia was significantly higher in GM compared to WM in both ASD ($F(1,16) = 13.582$, $p = 0.002$) and typically developing individuals ($F(1,14) = 13.371$, $p = 0.002$; Fig. 1F). Total densities (N/mm^3) of all Iba1-ir microglia phenotypes in both gray matter (GM) and white matter (WM) did not differ between ASD and typically developing individuals (GM; $F(1,15) = 0.62$, $p = 0.443$ and WM; $F(1,15) = 0.30$, $p = 0.592$; Fig. 1F).

3.2. Decrease in ramified microglial density and increase in primed microglial density

Iba1 immunohistochemistry revealed differences in microglial morphology across different subjects and between the ASD and control group. As described, six different microglial phenotypes were identified in Iba1-immunolabeled sections of human temporal cortex. All six phenotypes were observed in gray matter and white matter of both groups. We did not see localization of specific phenotypes in both gray matter and white matter. In gray matter, the density of ramified microglia (Fig. 2A) was significantly lower in ASD than typically developing individuals ($F(1,15) = 6.38$, $p = 0.023$)) and the density of primed microglia (Fig. 2B) was significantly higher in ASD than typically developing individuals ($F(1,15) = 9.07$, $p = 0.009$; Fig. 2C). A repeated measure ANCOVA with the two phenotypes showed a significant interaction between phenotype (ramified and primed) and diagnosis ($F(1,15) = 16.490$, $p = 0.001$). In white matter, the density of ramified microglia (Fig. 2D) was significantly lower in ASD than typically developing individuals ($F(1,15) = 4.65$, $p = 0.048$; Fig. 2F), but there was no difference in primed microglia ($F(1,15) = 3.290$, $p = 0.090$; Fig. 2E and F). The morphology of ramified and primed microglia may seem similar, but by quantifying morphological parameters (cell body area and circularity) of ramified and primed microglia, we confirmed that our classifications were not biased and consistent with the criteria we used (Fig. S1). Densities of other microglial phenotypes (Fig. 3 top panel) did not differ significantly between typically developing individuals and ASD in both gray matter and white matter (Fig. 3A and B).

3.3. Age-related changes in primed microglia

At around age 2–3 years, typically developing individuals and ASD donors showed rod and reactive microglia, but typically developing individuals show less of these two morphologies compared to ASD (Fig. 4 top panel). From age 8 to 32 years, typically developing individuals show more ramified microglia than primed microglia or other non-ramified microglia morphology. However, in ASD there are more primed microglia than ramified microglia compared to typically developing individuals (Fig. 4 top panel).

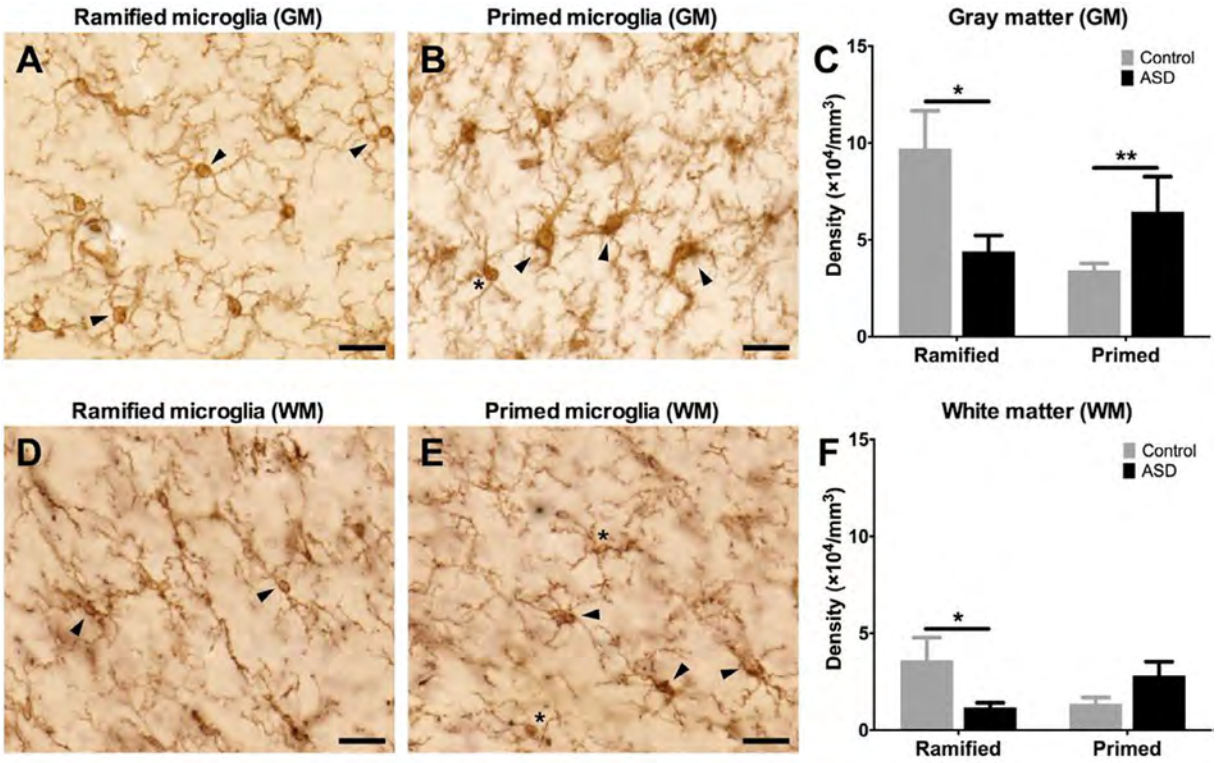

Fig. 2. Changes in density of ramified and primed microglia in both gray matter and white matter. Representative micrographs were taken at 20× objective from different subjects demonstrating different microglial phenotypes. In gray matter, (A) Ramified microglia (black arrowhead and asterisk in (B)) are characterized by a small round soma with fine processes branching out (control, 16.6 years; UMB-1322), (B) primed microglia (black arrow head) are characterized by an enlarged soma (sometimes oval shaped) and de-ramification (with thicker branch processes) (ASD, 9 years; AN01293), (C) density of ramified microglia in gray matter were significantly decreased in ASD, but primed microglia in gray matter were significantly increased in ASD. In white matter, morphology of the two phenotypes may seem slightly different from that of gray matter, but the characteristics of each phenotype remain the same. (D) Ramified microglia (black arrowhead and asterisk in (E); ASD, 12.9 years; UMB-4305), (E) primed microglia (black arrow head; ASD, 12.9 years; UMB-4305), (F) density of ramified microglia in white matter was significantly decreased in ASD, but primed microglia showed no significant difference between the two groups. Scale bar = 20 μm. $^{*}p < 0.05$ and $^{**}p < 0.01$.

In the ASD group, the correlation between age and density of primed microglia was significantly positive in both GM and WM (GM; $r = 0.45$, $p = 0.007$ and WM; $r = 0.364$, $p = 0.03$; Fig. 4 bottom panel). However, in the case of typically developing individuals, the correlation between age and density of primed microglia was not significant in either GM or WM (GM; $r = 0.444$, $p = 0.095$ and WM; $r = 0.333$, $p = 0.211$; Fig. 4 bottom panel). There were no significant correlations in other phenotypes (ramified, reactive, perivascular, rod, and dystrophic) with age in both groups (data not shown).

3.4. Cluster of microglia in gray matter and white matter

We additionally observed clusters of microglia in both gray matter (Fig. 5A and B) and white matter (Fig. 5C and D). These clusters of microglia have been observed in postmortem brains of multiple sclerosis (MS) and classified as a type of lesion (termed (p) reactive lesions in De Groot et al., 2001 and van Horssen et al., 2012; see also Boche et al., 2013). These clusters of microglia were found proximal to microvessels in the absence of vasculature damage or demyelination. Our brain samples did not have any lesions apart from these preactive lesions, so the percentage of lesions for preactive lesions couldn't be quantified like the two studies (De Groot et al., 2001; van Horssen et al., 2012). Instead, we qualitatively report that microglial clusters were present in both ASD and control, but more frequently observed (and more clusters within each subject) in ASD (8 out of 10 cases) than the control group (5 out of 9 cases) for both gray matter and white matter.

4. Discussion

To our knowledge, this is the first study quantitatively examining the distribution of different microglial phenotypes in postmortem human temporal cortex in both ASD and typically developing individuals. Using a stereological approach, our results show that there is a decrease in the density of ramified microglia, but an increase in primed microglia in ASD compared to typically developing individuals, while no change in overall microglial density. These results support our hypothesis that although there is no difference in the total number of microglial density, there are changes in selective microglial phenotypes. It is highly unlikely that there were any significant microglial morphological changes occurring postmortem (Dibaj et al., 2010; Eyo and Dailey, 2012; Torres-Platas et al., 2014b). Thus, one could assume that this microglial distribution in postmortem brain tissue reflects the local neuroimmune state at the time of death, as well as being indicative of immune challenges during the lifetime of an individual (Holtman et al., 2015). The shift in selective microglial morphology and distribution may serve as evidence for modeling specific microglial pathology to study the etiology and development of ASD.

Ramified microglia have fine processes branching out into the neuropil and capable of reacting to subtle microenvironmental changes arising from a variety of factors including pathogens, stress and tissue damage due to injury (reviewed in Davis et al., 1994; Streit et al., 1999). The role of ramified microglia has been suggested to be monitoring synaptic activity, synaptic plasticity and phagocytosis for fine-tuning neuronal networks (reviewed in

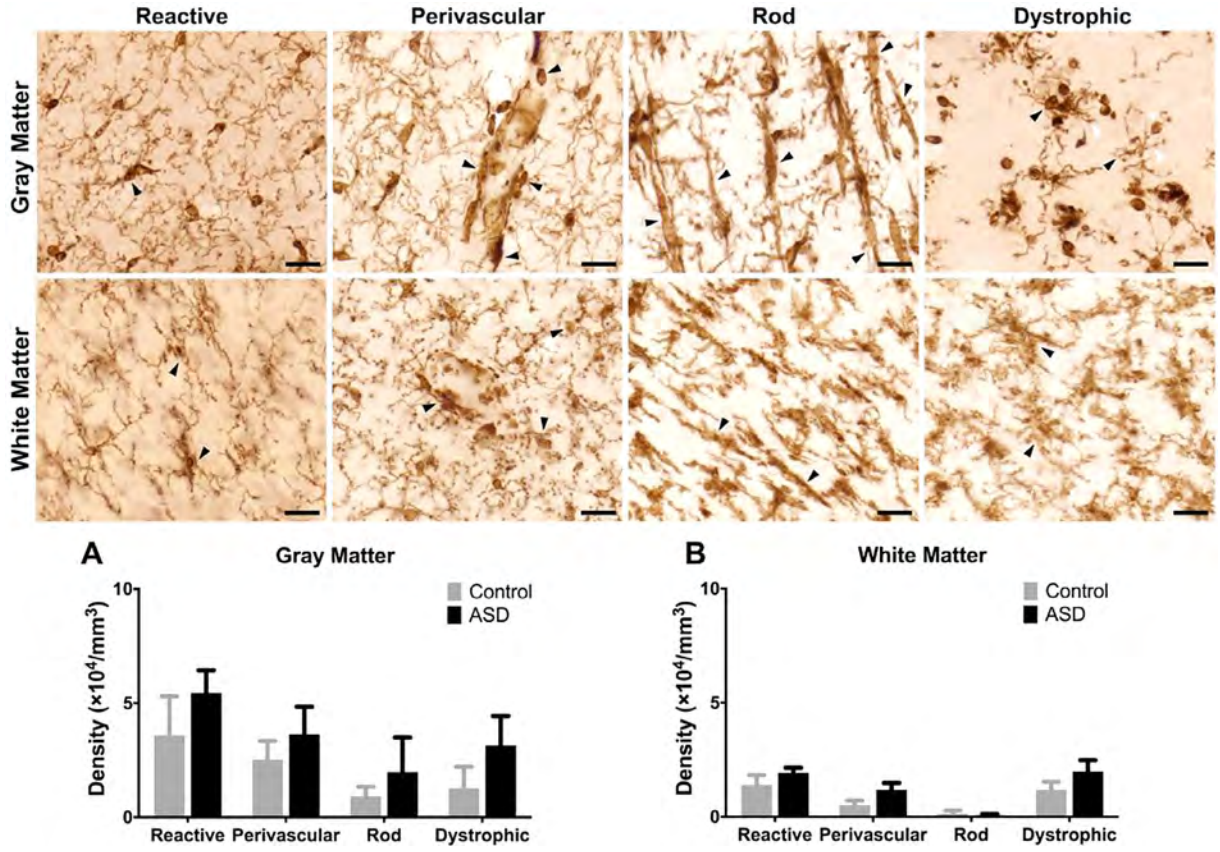

Fig. 3. No changes in density of perivascular, reactive, rod, and dystrophic microglia in both gray matter and white matter. Top panel: In gray matter, reactive microglia (black arrow head) are characterized by an enlarged soma with one or less branched processes (ASD, 23 years; IBR-93-01), perivascular microglia (black arrowhead) are amoeba shaped microglia/macrophages typically associated with blood vessels (ASD, 8.5 years; HSB-4640), rod microglia (black arrowhead) are characterized by a sausage-shaped soma and long, thin processes (ASD, 2.8 years; B-6399) and dystrophic microglia (black arrow head) are characterized by a partially de-ramified branch processes (white asterisks) with large swellings (spheroid) at the end or twisted and shortened branch processes (white arrow head) (ASD, 11.9 years; Cal-105). In white matter, reactive microglia (ASD, 12.9 years; UMB-4305), perivascular microglia (ASD, 8.5 years; HSB-4640), rod microglia (control, 2.1 years; BTB-4235) and dystrophic microglia (control, 2.1 years; BTB-4235) were identified using the same criteria. (A, B) In both gray matter and white matter the density of all microglia phenotypes did not show a significant different between the two groups. Scale bar = 20 μm.

Bilimoria and Stevens, 2015; Morris et al., 2013; Tremblay et al., 2011; Wu et al., 2015). Decrease in ramified microglial density in ASD may underlie the deficits in synaptic monitoring and pruning that leads to increased shorter and local connectivity, over-connected and redundant cortical networks in ASD (Solso et al., 2016; Whyte et al., 2015; Keown et al., 2013; Supekar et al., 2013; Courchesne et al., 2005; Zikopoulos and Barbas, 2010, 2013). Also, the impaired synaptic pruning may underlie the increased number of dendritic spines even after adolescence in postmortem ASD brains compared to typically developing individuals (Hutsler and Zhang, 2010; Tang et al., 2014). However, it is unclear whether this decrease in ramified microglia is due to an innate depletion as a result of a genetic predisposition and whether or not it directly relates to abnormal synaptic development. One possibility is the increase in microglial apoptosis, which suggests a potential role of autoimmunity in ASD (reviewed in Gesundheit et al., 2013). However, it is most likely that environmental factors during pregnancy and development lead to a shift into other morphologies, primarily to primed microglia.

Primed microglia are characterized by an average 2.5-fold increase in cell body size with no difference in branching patterns (but thicker primary branch processes) compared to ramified microglia (Torres-Platas et al., 2014a). With these morphological changes, there is an increased expression of cell-surface antigens such as MHC class II (Henry et al., 2009; VanGuilder et al., 2011) and CD11b/c (Stichel and Luebbert, 2007; Perry et al., 1993). An increase in IFN-γ (Cunningham, 2013), CNS and peripheral infections, chronic inflammation, psychological stress, and aging can induce microglial priming (reviewed in Niraula et al., 2016; Holtman et al., 2015; Norden and Godbout, 2013; Dilger and Johnson, 2008) that imprints microglia to become highly reactive to secondary insults. When there is a secondary insult, it triggers a hyper-reactive state of microglia characterized by secretion of excessive cytokines, chemokines, and other reactive molecules associated with neurotoxicity (Benedetto et al., 2003; Godbout et al., 2005; Onore et al., 2014; Barth et al., 2016). In turn, this increase in inflammatory cytokines could result in social and cognitive behavioral deficits that are relevant to core symptoms of ASD (reviewed in Meyer et al., 2009; Meyer, 2014; Gottfried et al., 2015; Nakagawa and Chiba, 2016).

Our results showed an increase in primed microglia in ASD compared to typically developing individuals. It has been shown that HLA-DR, an MHC class II cell surface receptor, is significantly increased in the cerebellum of ASD (medial frontal gyrus and anterior cingulate cortex was not significant, but the average was higher in ASD; see Vargas et al., 2005). Though HLA-DR in the temporal cortex has not been examined, together this supports that microglial priming is present in the ASD brain. This suggests that during development, individuals with ASD experience stressors which could prime the immune system and there are several stressors associated with ASD which may be involved. Infections (reviewed in Knuesel et al., 2014), exposure to obesogens

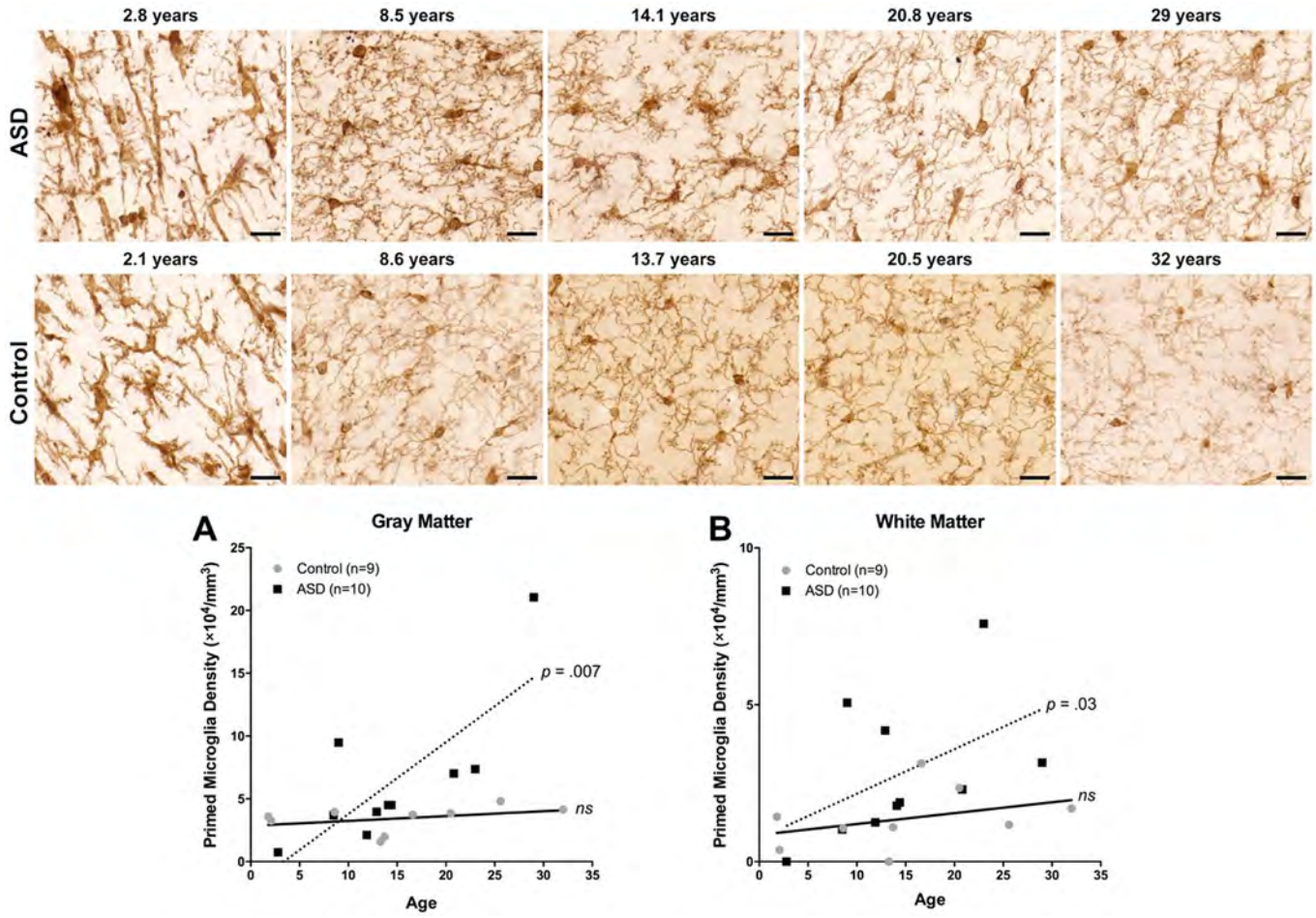

Fig. 4. Age comparison of Iba1-immunoreactive microglia in gray matter between typically developing individual (control) and ASD donors. Top panel: Micrographs were taken at 20× objective in temporal cortex gray matter from ASD and typically developing individual donors. In the ASD donors, there are more primed microglia observed than typically developing individuals, and more ramified microglia are observed in the typically developing individuals. Micrographs from ASD donors were taken from ages 8.5 years (HSB-4640), 14.1 years (UMB-4315), 20.8 years (UMB-4999) and 29 years (B-6994). Micrographs were taken from typically developing individual (control) ages 8.6 years (UMB-1706), 13.7 years (UMB-1790), 20.5 years (UMB-4590) and 32 years (IBR 291-00). Scale bar = 20 μm. Bottom Panel: A non-parametric rank correlation (Kendall's tau) between age and primed microglia density showed a significantly positive correlation in ASD (dotted trend line), but not typically developing individuals (solid trend line) in both gray matter ($r = 0.450$, $^{**}p = 0.007$; A) and white matter ($r = 0.364$, $^{*}p = 0.03$; B).

(reviewed in Wong et al., 2015; Guinchat et al., 2012; Boksa, 2010), stress (reviewed in Kinney et al., 2008), and neuroendocrine disruptions (Aiello and Whitaker-Azmitia, 2011; Whitaker-Azmitia et al., 2014) during pregnancy and development have been shown to be significant risk factors for ASD. In animal models of these risk factors, number of studies have shown evidence for these factors being involved in microglial priming and ASD-like behavior. In an animal model of maternal immune activation, offsprings showed exaggerated immune responses along with aggravated social and cognitive behaviors compared to offsprings that came from non-immune activated dams (Willette et al., 2011; Lucchina and Depino, 2014; Kazlauskas et al., 2016). Animals prenatally treated with a high-fat diet showed an exaggerated cytokine release and anxiety-like behavior when challenged with LPS compared to low-fat diet treated animals (Bilbo and Tsang, 2010). Animals prenatally exposed to diesel exhaust particles (DEP) showed altered microglia density and cell-surface antigen expression and exaggerated immune responses to a high-fat diet in adulthood compared to non-DEP exposed animals (Bolton et al., 2012, 2014).

It is, however, important to note that if microglial priming occurs during *development* due to a number of factors, its effect will be *permanent*. During perinatal and adolescence, the brain is highly plastic to environmental factors, and changes that occur during this critical period can permanently change the brain. Like the brain, which can be programmed during development through environmental factors, the immune system is also programmed during development. Therefore, disturbances during development can result in permanent structural and functional states of the CNS and immune system, which alter later-life neuroplasticity, neuronal function and behavior (Brenhouse and Schwarz, 2016). Microglial priming is a prime example of developmental programming of the neuro-immune system by which microglia are forever in a "primed" state during development and throughout the lifespan. The increase in primed microglia density as a function of age suggests a developmental effect on primed microglia density. It is possible when secondary insults occur, initially primed microglia initiate a cascade of microglial priming in neighboring microglia. However, with the limited number of subjects per age group, the exact interaction between age and primed microglia density remains unclear. Also, we did not see any differences in other phenotypes (reactive, perivascular, rod and dystrophic), between ASD and typically developing individuals, suggesting that the ASD brain is not under constant inflammation, but instead primed to react to any secondary insult.

The temporal cortex includes regions critical for auditory processing and social cognition such as the primary auditory cortex

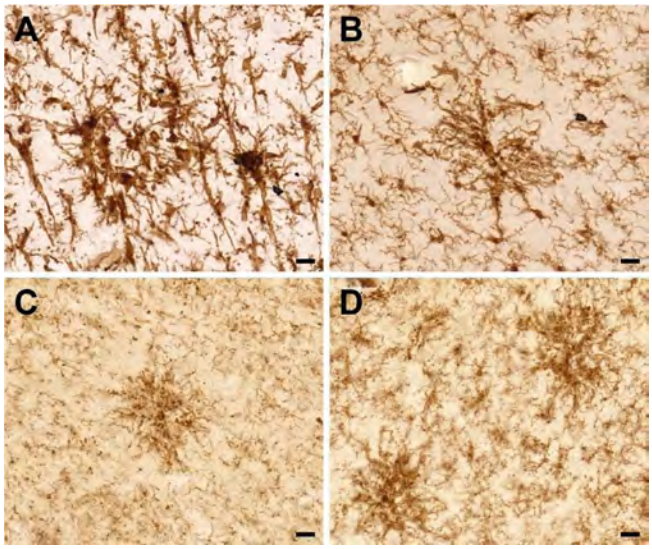

Fig. 5. Iba1-immunoreactive microglia cluster observed in gray matter and white matter. Micrographs were taken at 10× objective and represent clusters of microglia (also known as preactive lesions) in gray matter (A: 2.8 years, B-6399; B: 14.1 years, UMB-4315) and white matter (C and D, 9 years, AN01293) in individuals with ASD. Scale bar = 20 μm.

and fusiform face area. The decrease in ramified microglia may be contributing to the under-connectivity and structural changes shown in the ASD temporal cortex (Zilbovicius et al., 2000; reviewed in Courchesne and Pierce, 2005; Xiao et al., 2014), which may lead to deficits in auditory processing and social cognition (Bigler et al., 2007; Ventola et al., 2013). However, it is unlikely that microglial priming leads to a deficit in function of the temporal cortex, but rather increases the vulnerability to aggravation of its function. With increased microglial priming in this region, the detrimental effects of secondary insults (e.g., systemic inflammation) are directed to this region that is already vulnerable, thus exacerbating functions already impaired. For example, excessive cytokine release can lead to abnormal neurotransmitter activity that can lead to changes in synaptic transmission and plasticity (reviewed in Haroon et al., 2012) or develop neuropsychiatric comorbidities (Gjevik et al., 2011; Gotham et al., 2015; Joshi et al., 2010; Leyfer et al., 2006).

In addition, we observed microglial pathology that has not been previously reported in the postmortem ASD literature. Clusters of microglia were observed in both white matter and gray matter of typically developing individuals and ASD, but the frequency was higher in ASD. It has been suggested that the cluster of microglia is not associated with any blood-brain-barrier related damage or astrogliosis, but an innate and intrinsic immune activation (De Groot et al., 2001; van Horssen et al., 2012). It is, however, difficult to suggest innate immune activation in ASD in our samples, but the higher occurrences of these clusters are suggestive of a dysfunction in the innate immune system in ASD, which have been previously examined (Jyonouchi et al., 2002; reviewed in Gesundheit et al., 2013).

Overall, our results overall provide evidence to developmental microglial priming in ASD. This brings light to potential targets for diagnostic methods and pharmacological interventions. As post-parturition exposure to pathogens is inevitable, prevention of microglial activation through pharmacological interventions may be beneficial. Though there are not many studies, prevention of microglial activation through pharmacological interventions (e.g., minocycline) rescued increased neuroinflammation and spatial memory impairment (McKim et al., 2016), decreased mother-pup bonding (Miyazaki et al., 2016), and decreased social interaction (Kumar and Sharma, 2015). While simply inhibiting microglial activation at the point of exaggerated immune response using minocycline may help, it may not necessarily prevent microglial priming if treated during pregnancy and development. Treating minocycline prenatally or perinatally can actually lead to increased cell death and microglial density in mice (Strahan et al., 2016) and rodents (reviewed in Inta et al., 2016). However, it is important to note that ASD is a spectrum and that not all individuals with ASD will have microglial priming. That is, only a subset of ASD will show an exaggerated response to secondary immune insults associate with changes in behavior (Jyonouchi et al., 2014), which should be considered during diagnoses and treatment.

As many postmortem studies may indicate, there are limitations in terms of our speculations. First, though there are several excellent brain banks available, there are limitations to obtaining suitable numbers of brain samples of diseased patients. Second, there are limited number of suitable control brain donors with no known psychiatric diagnoses. Third, due to the variation in cutting and sectioning of the brain, proper identification of regional anatomy is needed. Fourth, the postmortem interval (PMI) and storage time in formalin can influence the quality of the immunolabeling of cells, but we have controlled for these factors in our statistical analyses. In this paper, brain samples were obtained from a variety of brain banks, and studied while encoded to minimize experimenter bias.

While there are limitations to postmortem studies, it still remains a very valuable and unique source of cellular examination of neuropsychiatric disorders and neurological diseases. Our results provide new insights to the microglial pathology in ASD with an emphasis on examining individual microglial phenotypes. Most studies regarding microglial priming come from studies of aging humans and animals (Perlmutter et al., 1992; Sheng et al., 1997; VanGuilder et al., 2011; Perry et al., 1993), and there are not enough animal models investigating microglial priming and ASD. Future studies should investigate the relationship between maternal immune activation (through systemic inflammation, stress, diet, drug treatment, genetic predispositions, hypoxia-ischemia, diet, parental age, etc.), microglial priming, and behavioral changes parallel to ASD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2017.01.019.

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