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Sex Differences in Early Postnatal Microglial Colonization of the Developing Rat Hippocampus Following a Single-Day Alcohol Exposure

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Abstract

Microglia are involved in various homeostatic processes in the brain, including phagocytosis, apoptosis, and synaptic pruning. Sex differences in microglia colonization of the developing brain have been reported, but have not been established following alcohol insult. Developmental alcohol exposure represents a neuroimmune challenge that may contribute to cognitive dysfunction prevalent in humans with Fetal Alcohol Spectrum Disorders (FASD) and in rodent models of FASD. Most studies have investigated neuroimmune activation following adult alcohol exposure or following multiple exposures. The current study uses a single day binge alcohol exposure model (postnatal day [PD] 4) to examine sex differences in the neuroimmune response in the developing rat hippocampus on PD5 and 8. The neuroimmune response was evaluated through measurement of microglial number and cytokine gene expression at both time points. Male pups had higher microglial number compared to females in many hippocampal subregions on PD5, but this difference disappeared by PD8, unless exposed to alcohol. Expression of pro-inflammatory marker CD11b was higher on PD5 in alcohol-exposed (AE) females compared to AE males. After alcohol exposure, C-C motif chemokine ligand 4 (CCL4) was significantly increased in female AE pups on PD5 and PD8. Tumor necrosis factor- α (TNF- α) levels were also upregulated by AE in males on PD8. The results demonstrate a clear difference between the male and female neuroimmune response to an AE challenge, which also occurs in a time-dependent manner. These findings are significant as they add to our knowledge of specific sex-dependent effects of alcohol exposure on microglia within the developing brain.

Keywords

Immune; FASD; Postnatal brain; Microglia; Sex differences

Introduction

Microglia have been linked with the brain's inflammatory response to insult, including drug exposure, stress, and infection. Microglia strive to maintain homeostasis whether the brain is

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under assault by teratogens or physical trauma, and also during normal conditions through constant surveillance, maintenance of synapses and circuits, and self-renewal (Ajami et al. 2007; Aguzzi et al. 2013; Wake et al. 2013). In the healthy developing brain, microglia are involved in many necessary, normal development processes including the culling of apoptotic cells, synaptic pruning, and masculinization of the brain and behavior (Dalmau et al. 1998; Bessis et al. 2007; Paolicelli et al. 2011; Lenz et al. 2013). Following identification of a pathogen, microglia respond by changing their phenotypic activation state to increase production of pro-inflammatory cytokines (Kreutzberg 1996; Hanisch 2002; Town et al. 2005; Neumann et al. 2008; Kane et al. 2011; McClain et al. 2011; Topper et al. 2015; Boschen et al. 2016).

Alcohol is one of the most commonly abused substances with teratogenic properties and has been well-characterized over the past few decades. Currently, prenatal alcohol exposure is the leading preventable cause of birth defects and intellectual disabilities in the United States, with alcohol-exposed pregnancies recently estimated to affect 2.4–4.8% of all live births in US (Stratton et al. 1996; Bailey and Sokol 2008; May et al. 2014). Alcohol-exposure during pregnancy can lead to the development of Fetal Alcohol Spectrum Disorders (FASD). FASDs encompass a variety of physical and cognitive symptoms that affect the exposed individual throughout their lifetime, with fetal alcohol syndrome (FAS) being the most severe. Later-developing brain structures, including the hippocampus and prefrontal cortex, are especially vulnerable to the teratogenic effects of alcohol during the third trimester of pregnancy (Goodlett et al. 1990; Bonthius and West 1991; Mooney et al. 1996; Ikonomidou et al. 2000; Klintsova et al. 2007). Equivalent developmental events in the brain occur during the first two postnatal weeks of life in altricial rodents, including rats and mice (Dobbing and Sands 1979; Semple et al. 2013). It has been shown in rodent models of FASD that even a single day of alcohol exposure during the brain growth spurt, a period characterized by a surge in neuronal proliferation, migration, and synaptogenesis, can induce brain weight reduction and neuronal cell loss, especially in the cerebellum and forebrain structures (Dobbing and Sands 1979; Goodlett et al. 1989; Goodlett et al. 1990). Alcohol exposure during the third trimester-equivalent has been shown to induce hippocampal apoptosis, and likely signals microglia through release of chemoattractant molecules (Ahlers et al. 2015). Recent work in our lab (Boschen et al. 2016) reported that binge-like alcohol exposure during the third trimester-equivalent increased microglial activation and production of pro-inflammatory cytokines and the anti-inflammatory cytokine transforming growth factor- β (TGF- β) in the developing rat hippocampus. Microglia are responsible for clearing apoptotic cellular debris through phagocytic mechanisms (Ikonomidou et al. 2000; Jang et al. 2002; Petersen and Dailey 2004; Sierra et al. 2010). If aberrant microglial activation occurs, microglia can become persistently primed, leading to exaggerated immune responses following further challenges or neurodevelopmental or psychopathological disorders, such as schizophrenia and depression (Cai et al. 2000; Meyer et al. 2006; Miller et al. 2009; Monji et al. 2009).

The impetus to study sex-specific teratogenic effects on neurodevelopment has grown as evidence mounts that males and females differ in normal developmental processes in early gestation. For example, studies by Schwarz et al. (2012) found that microglial colonization of the brain during the early neonatal period differs between male and female rats, and

Gilmore et al. (2007) found differences in neonatal, regional gray matter growth between male and female infant children. In addition, sex-specific differences in susceptibility to adverse outcomes caused by developmental exposure to stress or drugs, including nicotine, isoflurane, and alcohol have been reported for many years (Goodlett and Peterson 1995; Slotkin et al. 2007; Lupien et al. 2009; Schwarz et al. 2012; Lee et al. 2014). Thus, it has become imperative to investigate whether microglia respond to teratogens, such as alcohol, in a sex-dependent manner.

The goal of the current study was to examine sex-specific changes in microglial number at two time points (postnatal days [PD] 5 and PD8) during the development of the rat hippocampus, as well as the sex-specific immune response following a single-day binge alcohol exposure on PD 4. A single day of alcohol exposure in rats mimics a fetus exposed to binge-drinking over one weekend during the third trimester of pregnancy and is relevant to the human condition, as a recent study reported that 1 in 33 women admitted binge-drinking during pregnancy (Tan et al. 2015). Subregion-specific microglial counts and expression of five genes that code for pro-inflammatory (interleukin-1 β [IL-1 β], C-C motif chemokine ligand 4 [CCL4], tumor necrosis factor- α [TNF- α], and cluster of differentiation molecule 11B [CD11b]) or anti-inflammatory (TGF- β) markers (cytokines, chemokines, or cell surface receptors) were measured to assess both the number of microglial cells throughout development and level of activation of microglia in the hippocampus either 24 or 96 h post-alcohol exposure. The above classical markers of inflammatory response were chosen for this study as they have all been shown to be affected by immune insults in similar lines of research (McClain et al. 2011; Marshall et al. 2013; Topper et al. 2015; Boschen et al. 2016). Developmental and neuroimmune sex differences were analyzed both at baseline (in controls without alcohol exposure) and in response to alcohol.

Experimental Procedures

Animals

Timed-pregnant Long-Evans rat dams were acquired from Harlan Laboratories (Indianapolis, IN). Dams were singly housed in the University of Delaware Animal Care facility with ad libitum access to food in cages of standard dimensions (17 cm high $\times$ 145 cm long $\times$ 24 cm wide) in a 12/12 h light cycle (lights on at 9:00 AM) upon arrival. On PD3, each litter was culled to ten pups (5 males, 5 females when possible). On PD4, pups were randomly assigned to one of three experimental groups: suckle control (SC), sham-intubated (SI) or alcohol-exposed (AE), using a split-litter design so that AE and SI animals were represented in the same litter. Following the binge-alcohol exposure procedure, pups were left undisturbed with the dam until sacrifice on PD5 or PD8. Both male and female pups were used for analyses for this current project. A total of 146 animals from 31 litters were used for the data (both immunohistochemistry and gene expression) presented here (Table 1). All procedures were carried out in accordance with the animal use protocol approved by University of Delaware Institutional Animal Care and Use Committee and in accordance with NIH's Animal Care Guidelines.

Alcohol Exposure Paradigm

AE pups received ethanol in a milk formula through intragastric intubation according to a widely used model for binge-exposure (2 feedings, 2 h apart, total dose 5.25 g/kg in an 11.9% vol/vol solution) on PD4. Specifically, pups received two alcohol/milk feeding followed by two milk-only feedings, to account for potential nutrients lost by missed suckling from the dam. Rats in the SI condition were intubated using the same procedure as the AE pups to control for the possible stress effects caused by the intubation procedure, but received no liquid solution, as extra milk dosing has been shown to abnormally accelerate the growth of SI pup (Goodlett and Johnson 1997; Goodlett et al. 1998). SC pups remained undisturbed with the dam except for daily weight measurements to ensure healthy development.

Blood Alcohol Concentrations (BACs)

On PD4, 90 min post-second-intubation blood samples from both the AE and SI groups were obtained through tail clippings and collected into heparinized tubes (< 60 µl per animal) to determine the blood alcohol concentrations. Blood was collected from the SI group to account for stress on the AE group, but discarded after collection without BAC analysis. Samples from the AE group were centrifuged (15,000 rpm/25 min) and the plasma collected and stored at -20 °C until analysis. Plasma was analyzed for BAC using an Analox GL5 Alcohol Analyzer (Analox Instruments, Boston, MA).

Immunohistochemistry

On PD5 or PD8, animals were deeply anesthetized with injections of ketamine/xylazine cocktail and subsequently transcardially perfused with heparinized 0.1 M phosphate-buffered saline (PBS; pH 7.2) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.2). Brains were stored in 4% paraformaldehyde overnight, then transferred to 30% sucrose in 4% paraformaldehyde for approximately one week until sectioning (switched to fresh solution twice during the week to ensure maximum tissue penetration). Brains were sectioned horizontally at 40 µm through the entire hippocampus and stored at -20 °C in cryoprotectant solution (ethylene glycol, 300 mL/1 L, and sucrose, 300 g/1 L in 0.1 M PBS). Sections were selected for processing using a systematic random sampling procedure (1:12). Microglia were identified by immunohistochemistry using an antibody against the microglial-specific marker ionized calcium-binding adaptor molecule 1, Iba-1 (Wako Chemicals, Richmond, VA, USA; 1:5000; Fig. 1). Positive staining was visualized with diaminobenzidine (DAB) labeling, as described in our previous study (Boschen et al. 2016).

Cell Quantification

Counts of Iba-1+ cells were made in an unbiased manner within a known volume of the dentate gyrus, CA1, and CA3 regions of the hippocampus (Fig. 1a), using the optical fractionator probe (Stereo Investigator, MBF Bioscience., Williston, VT). Each region was divided into three layers by tracing as follows: 1) for dentate gyrus (DG): granule cell layer (GCL), hilus, and molecular layer; 2) for CA1 and CA3 regions: pyramidal cell layer (PCL), stratum oriens, and stratum radiatum. The StereoInvestigator software calculates the total volume of the outlined brain region taking into consideration the total number of sections

(section sampling fraction, $ssf = 1/12$) within the structure of interest and the number of the sampling sites within the brain regions of interest on each section. The sampling grid and counting frame were set to 200 X 200 μm . Section thickness fraction was estimated as the ratio between dissector height and tissue thickness measured at every 5th sampling site. A dissector height of 12 μm and a guard zone of 2 μm on either side of the section were used. The mean coefficient of error (CE) for the number of cells did not exceed the recommended $m = 0.1$ (Gundersen 1986).

Gene Expression Assays

Following rapid decapitation on PD5 and PD8, brains were frozen with -20° 2-methylbutane and sectioned into 1 mm sections on a brain matrix. The sections were mounted onto slides which were rapidly frozen on dry ice and stored at -80°C . The hippocampus was dissected from the slide on dry ice and homogenized in the lysis buffer RLT Plus (Qiagen Inc., Valencia, Calif., USA). RNA was extracted from the target tissue using the AllPrep DNA/RNA Mini Kit (Qiagen Inc., Valencia, Calif., USA) and stored at -80°C . Nucleic acid concentrations were analyzed using spectrophotometry (NanoDrop 2000, ThermoScientific). cDNA was synthesized from the RNA using the Quantitect Reverse Transcription kit (Qiagen Inc., Valencia, Calif., USA). Gene expression was assessed for a total of 76 animals using real-time PCR (Bio-Rad CFX96) with Taqman probes (ThermoFisher Scientific) for the following target genes: *Il1b*, *Ccl4*, *Tnf*, *Itgam*, and *Tgfb1* (Table 2). *Tubulin* was used as a reference gene. All reactions for each gene target and reference were run in triplicate. Product specificity of the amplicon was assessed using ethidium bromide-agarose gel electrophoresis. The comparative Ct method was used to obtain the relative fold change in gene expression of experimental (AE) vs. the average of controls within each sex per plate (Livak and Schmittgen 2001).

Statistical Analysis

For PD 4, 5, and 8 weight assessment, repeated-measures ANOVAs (Day x Treatment) were performed to compare weights of animals within neonatal conditions on those days. Males and females were not separated for weight analyses as there are no significant differences during this developmental timeframe. The average BAC for each animal (2–3 analyses/animal) is reported as $\text{mg/dl} \pm$ standard error of the mean (SEM).

To determine the impact of neonatal alcohol exposure on microglial number and activation stats, initial ANOVAs were performed that included all three treatment groups separately. However, as no significant differences were seen between SI and SC animals on any measure, these groups were combined into a single control group to increase statistical power. This approach is in concordance with other literature from our lab and others (Otero et al. 2012; Hamilton et al. 2015). For microglial number, two-way ANOVAs were performed with the factors of Sex by Treatment within each time point. When appropriate, Sidak's test of multiple comparisons was run. Change in microglial number across days was analyzed using two-way ANOVAs (Treatment x Day) within each sex. For gene expression, two-way ANOVAs (Sex x Treatment) were for each time point followed by appropriate post hoc tests. All data are expressed as mean $\pm$ standard error of the mean (SEM) except where

otherwise noted. Statistical significance was set as $p < 0.05$. Trends towards significance ($p < 0.1$) are discussed when appropriate.

Results

Animal Weights and Blood Alcohol Concentrations (BACs)

All animals were weighed on PD4 (before intubation) and PD5 or 8 (before sacrifice) (Table 3; Fig. 2). A repeated-measures ANOVA revealed that the pups gained significant weight between PD4 and PD5 and between PD4 and PD8. Between PD4 and PD5, a significant interaction of Day and Treatment was found, however post hoc analyses (Sidak's) were not significant. There was no main effect of Treatment on weight on any day. Table 2 shows the average weights for each condition at each age. BACs were analyzed 90 min following the second alcohol administration and were found to be 288 ± 7.21 mg/dL.

Iba-1+ Microglia Cell Counts

Microglial number in the hippocampus was determined using unbiased stereological analysis of Iba-1+ cells. Two-way ANOVAs (Sex x Treatment) were run for each subregion (dentate gyrus (DG): molecular layer, granule cell layer (GCL), hilus; CA1: stratum oriens (SO), pyramidal cell layer (PCL), stratum radiatum (SR); CA3: SO, PCL, SR) (Fig. 1). On PD5, males had more microglia than females (Fig. 3) in the following brain regions: DG molecular layer, GCL, CA1 SO, CA1 PCL, CA1 SR, CA3 SO, CA3 PCL, and CA3 SR (Fig. 3a, b, d-i; Table 3). In addition, male AE pups were found to have had significantly more microglia compared to control and AE females on PD5 in the DG molecular layer and GCL (Fig. 3a and b). Male control pups also had more microglia than AE females in the molecular layer (Fig. 3a). Significant interactions of Sex x Treatment were observed in the molecular layer, GCL, and hilus (Fig. 3a-b). *Post hoc*s were not significant in the hilus. No main effect of neonatal treatment was found for any subregion.

On PD8, analysis of significant statistical interactions revealed that in the DG GCL, female AE pups had fewer microglia compared to controls (Fig. 4b). In the CA3 SO, AE females and male control rats had fewer microglia compared to female controls (Fig. 4g). Male controls also had fewer microglia in the PCL compared to female controls (Fig. 4h). While significant interactions of Sex x Treatment were found for the molecular layer, GCL, CA1 PCL, CA3 SO, CA3 PCL, and CA3 SR (Fig. 4a-b, e, g-i), significant *post hoc*s were only found for CA3 SO and PCL (Fig. 4g-h) and the DG GCL (Fig. 4b). No main effects of Sex or Treatment were found for any of the areas on PD8.

To determine if microglia number in the different hippocampal subregions changed across the days from PD5 to PD8, two-way ANOVAs (Treatment x Day) were performed for males and females separately (data shown in supplemental figures Online Resources 1 and 2). In all regions, female pups had more microglia in the hippocampus on PD8 than on PD5: DG molecular layer, GCL, hilus, CA1 SO, CA1 PCL, CA1 SR, CA3 SO, CA3 PCL, and CA3 SR (Online Resource 1A-I). In the DG subregions, AE females had fewer microglia on both days compared to control female pups: molecular layer, GCL, and hilus (Online Resource 1A-C). No significant interactions were found. In males, there were more microglia on PD8

than on PD5 in the hilus only (Online Resource 2C). It is possible that increased power through higher group numbers would have revealed more significant effects of day, as multiple regions (GCL, CAL PCL, CA1 SR, and CA3 SR) displayed a trend towards a main effect of Day ($p = 0.05$ – 0.08 in all cases). Overall, these data demonstrate that a significant number of microglia are being added to the hippocampus in the female brains during the early postnatal period while this effect is less pronounced in male pups.

Pro- and Anti-Inflammatory Cytokine Gene Expression

Expression of the pro-inflammatory cytokine and chemokine genes IL-1 β , CCL4, TNF- α , and pro-inflammatory marker CD11b, as well as anti-inflammatory TGF- β , were analyzed in the hippocampus on PD5 and PD8 (Table 3). Significant changes in IL-1 β , CCL4, and CD11b were discovered in the PD5 hippocampus (Fig. 5a). Notably, AE animals had increased IL-1 β compared to controls on PD5 with no differences between the sexes. For CCL4 expression, there was a main effect of Treatment in that AE animals had elevated CCL4 compared to controls and females had higher expression than males. However, this effect was driven by the fact that female AE pups had significantly higher expression compared to males and control females. For CD11b, females had higher expression in the hippocampus compared to males. No main effects or interactions were found for TNF- α or TGF- β on PD5. Two-way ANOVAs (Gene x Sex) determined that control males and females did not differ in baseline expression of any of the genes on PD5 (Gene: $p = 0.996$, Sex: $p = 0.83$, Interaction: $p = 0.99$) (data not shown). This finding corresponds to previous literature reporting no differences in cortical CCL4 and CD11b gene expression between male and female rat pups on PD4 (Schwarz et al. 2012).

For PD8, significant Sex x Treatment interactions were discovered for the genes CCL4 and TNF- α (Fig. 5b; Table 3). Female AE pups had significantly higher expression of CCL4 but downregulated expression of TNF- α compared to AE males and control females on PD8. Main effects of Sex were found for the genes CCL4 and TNF- α , those these effects were due to the robust expression changes in the AE females. Similarly, the AE female group drove a main effect of Treatment for CCL4. No interactions or main effects were found for the other genes. Overall, these results indicate that CCL4 is affected by both postnatal alcohol and sex of the pup in that AE females had significantly altered CCL4 expression compared to both male AE pups and female controls at both time points. IL-1 β and CD11b were only altered 24 h after alcohol exposure and had returned to normal levels by PD8. However, on PD8, TNF- α was only altered on PD8, suggesting a delay in changes to this cytokine. Interestingly, in contrast to our previous study (Boschen et al. 2016), TGF- β was not affected at either time point, suggesting that a longer exposure paradigm is necessary to upregulate anti-inflammatory signaling. Two-way ANOVAs determined that control males and females did not differ in baseline expression of any of the genes on PD8 (Gene: $p = 0.99$; Sex: $p = 0.923$, Interaction: $p > 0.999$).

Discussion

The goal of this study was to investigate sex differences in early postnatal microglial colonization in the developing hippocampus following a single day binge of alcohol

exposure during the third trimester-equivalent (PD4). Male and female rat pups differed significantly in the number of microglia in the hippocampus. Specifically, male control pups had more microglia in hippocampus compared to female controls on PD5 (Fig. 3), however, the number of microglia in males and females did not differ on PD8 as females added a significant number of microglia to all subregions of the hippocampus during this period (Fig. 4 and Online Resource 1). Males and females also showed different microglial response patterns following PD4 alcohol exposure. While postnatal alcohol exposure did not affect the number of microglia in the hippocampus of male rats at either time point (compared to controls), a decrease in microglial number was found in female rats in two hippocampal subregions on PD8 specifically. Additionally, neuroimmune activation (as measured with cytokine gene expression) was altered on both PD5 and PD8 in a sex- and cytokine-specific manner (Fig. 5). Overall, these results demonstrate that male and female rats have differing baseline levels of microglia during the early postnatal period that can rapidly change across days. Furthermore, while a single day binge-like dose of alcohol does not alter the number of microglia in the male hippocampus, this study is consistent with our previous work (Boschen et al. 2016) demonstrating a pro-inflammatory response with developmental AE. Data here also extend our previous work (6 days of AE) showing a pro-inflammatory response with just a single day binge-like exposure. The number of microglia may not necessarily correlate to the level of neuroimmune response in a given region, as other cells such as neurons and astrocytes can contribute to the release of cytokines as well (Lieberman et al. 1989; Aloisi et al. 1992; Gao et al. 2000).

Microglia play a crucial role in phagocytosis and removal of apoptotic cells and cellular debris during development. Colonization of the brain occurs in a region-specific manner; for example, the developing dentate gyrus is devoid of microglial precursors until around the time of birth (Dalmau et al. 1998). At birth, resident microglia develop short, thick processes that lengthen during the first postnatal week (Schwarz and Bilbo 2012; Schwarz et al. 2012). The natural, ramified state that persists through adulthood is not fully reached until rat adolescence (Cuadros and Navascués 1998; Schwarz and Bilbo 2012). In our study, control males had more microglia than control females in all hippocampal regions on PD5 (except for the hilus) (Fig. 3). However, by PD8, female and male controls largely did not differ in microglial number as females gained significant numbers of microglia in all subregions (Fig. 4 and Online Resource 1). In fact, female controls had more microglia in CA3 PCL than males on PD8. Our study supports previous research reporting that neonatal male rats have more microglia in the hippocampus during the early postnatal period (Schwarz et al. 2012). However, females have been found to have more microglia during adolescence and adulthood (Schwarz et al. 2012); it not known when exactly this switch occurs but, taking the current data into consideration, it likely happens during the later neonatal period. Finding sex-specific differences in timing and location of microglia colonization and presence may be critical to understanding the neuroendocrine-immune axis in both health and disease, as proposed by Schwarz and Bilbo (2012). The authors suggest that these differences in developmental windows may explain observed sex-biases in the onset and likelihood of neuropsychiatric disorders. In the case of FASD research, the differences in developmental windows may also account for sex-differences in time- and location-dependent susceptibility to alcohol-induced inflammation and damage. Sex-specific effects were also observed in

cytokine levels, as control female rats had higher levels of CD11b compared to males at PD5 (Fig. 5). Although female rat pups showed a decrease in microglia cell counts at PD5, this would not necessarily result in a corollary decrease in CD11b. Past studies have shown that CD11b antibodies may attach to marrow-derived dendritic cells and neutrophils as well as microglia (Fischer & Reichmann 2001; Matsumoto et al. 2007). The increase found in this study likely indicates an increase in neuroinflammation, and a morphological assessment of the microglia would be useful in determining if increased expression of CD11b correlates with a more activated phenotype at this time point.

The current study found that male AE pups had significantly more microglia compared to AE females in the DG molecular layer and GCL 24 h following the single-day binge-like alcohol exposure, suggesting that the initial neuroimmune response to neonatal alcohol exposure differs in male and female hippocampus (Fig. 3). On PD8, female AE pups had fewer microglia compared to controls in the GCL and CA3 stratum oriens (Fig. 4). The reasons for the observed sex-specific effects on microglial number in AE animals remain to be elucidated. One contributing factor could be region-specific differences in the timing of microglia colonization between males and females combined with preferential targeting of certain brain structures by third trimester-equivalent alcohol exposure. For males, it is possible that the activation of microglia could follow a pattern noted by Ahlers et al. (2015), where activation following acute alcohol exposure in adulthood was transient; microglia rapidly returned to a baseline state of activation within 24–48 h of exposure, subsequent to the elimination of apoptotic bodies. Rapid deactivation of microglia following a single-day alcohol exposure could explain why alcohol-related changes in microglial number were not observed in PD5 or PD8 males. The reduction in microglial number in AE females vs. controls observed on PD8 could be due to AE females not adding as many microglia between PD5 and PD8 compared to control animals, though this possibility was not borne out statistically. Further studies are needed to determine if AE disrupts the natural colonization process of the neonatal brain. Alternatively, the microglia normally present in hippocampus following initial colonization could have been recruited to other regions of the brain more adversely affected by alcohol exposure. The decrease in microglial number in females on PD8 is similar to the decrease in microglial number observed in the DG and CA1 regions of the male rat hippocampus on PD10 following six days of binge-alcohol exposure (PD4–9) (Boschen et al. 2016). While this decrease contrasts reported increases in number of microglia found in the male adult and adolescent brains of alcohol-exposed rats (McClain et al. 2011; Marshall et al. 2013), Kane et al. (2011) reported decreases in microglial number in the mouse cerebellum after PD3–5 AE and compromised microglia viability in mouse cell cultures exposed to alcohol. Thus, a decrease in microglia following AE is not completely surprising.

The pro-inflammatory cytokines IL-1 β , TNF- α , and CCL4, and the microglial activation marker CD11b, are all classical markers of microglial activation, whereas TGF- β has anti-inflammatory properties (Roberts and Sporn 1993). The current study found that PD4 alcohol exposure altered cytokine gene expression in the neonatal hippocampus in a sex-specific and timing-dependent manner (Fig. 5). Specifically, AE pups had significantly higher pro-inflammatory IL-1 β expression compared to controls on PD5, which could be cytotoxic with prolonged overexpression, but levels returned to normal by PD8. A previous

study by Chao et al. (1995) found that in culture, the combination of IL-1 β and TNF- α was synergistic and led to NMDA-receptor-related neurotoxicity. TNF- α was not affected by AE when measured on PD5 but showed significant upregulation in both male and female AE pups on PD8, highlighting the temporally-specific nature of the neuroimmune response. While TNF- α is a classical marker of neuroinflammation, upregulation can have both neurotoxic as well as neuroprotective downstream effects. Membrane-bound TNF receptor p55 contains a death-domain, which upon activation can lead to apoptosis, but receptor p75 activation typically leads to cell growth and survival (Kraft et al. 2009). The final outcome of TNF- α activation is complex, with some damaged neurons likely undergoing apoptosis, while others may be aided for survival. Expression of the chemoattractant molecule CCL4 was consistently upregulated in AE females at both time points: on PD5, female AE pups had higher CCL4 expression compared to AE males, as well as had higher levels compared to both control females and AE males on PD8. Sex by treatment interactions in CCL family expression were also reported following prenatal alcohol exposure (Terasaki and Schwarz 2016), with female embryonic brain tissue showing an exaggerated CCL2, CCL5, and CCL9 response following alcohol exposure compared to controls and male alcohol-treated embryos. These results, combined with the increase in females reported in the current study, support that the CCL family is specifically activated in females following developmental alcohol exposure. The implications of this sex-specific increase on risk or resiliency of female offspring to alcohol-related damage needs to be investigated. Interestingly, TGF- β was not affected by AE at either time point, in contrast to our previous report of increased levels following PD4–9 AE. Recent work by Topper et al. (2015) also reported no changes in TGF- β expression in the male rat hippocampus following a PD3–5 alcohol exposure. It is possible that a more prolonged exposure to alcohol is needed to elicit a TGF- β response. It is important to note that astrocytes can release cytokines as well (Lieberman et al. 1989; Aloisi et al. 1992); thus, the observed change in overall immune activation may not be entirely microglia-derived. Further work is needed to determine the contribution of astrocyte-mediated cytokine release in the current results.

These experiments support the importance of studying both males and females in developmental toxicology studies, as even in late gestation and the early neonatal period, significant physiological differences are apparent. More specifically, these differences indicate that males and females exposed to an equivalent dose of alcohol and during the same time period may differ in both the severity and location of effects exhibited. Under typical development conditions, males have been found to have significantly higher levels of testosterone than females from gestational day (GD) 18 until PD5, but up until GD18 females show the same levels as males (Weisz and Ward 1980). Prenatal alcohol exposure blocks the “testosterone surge” in males on GD18–19 and interferes with the sexual differentiation of the male brain; no effects on testosterone levels were found in females (McGivern et al. 1988; McGivern et al. 1993; Ward et al. 2003). Sex differences in prenatal alcohol-induced deficits in performance on hippocampal-associated tasks have also been reported. Kelly et al. (1988) found that prenatal alcohol led to impaired spatial navigation only in female rats, while Zimmerberg et al. (1991) demonstrated impaired working memory in male rats after prenatal AE. These studies show that alcohol administered during the prenatal period alters behavior in a sex-dependent manner into

adulthood. Gonadal hormones such as testosterone, progesterone, and estradiol can influence reactive gliosis by altering the number of reactive microglia and associated cytokines released following acute trauma (Arevalo et al. 2013). If sex-specific hormones can affect microglial function in response to physical insult, they may also be involved in driving sex-specific responses to developmental alcohol exposure. Prenatal AE can prime the immune system to an exaggerated response when given a “second-hit” immune challenge in adulthood (Terasaki and Schwarz 2016, 2017). Both LPS and acute alcohol exposure elicited an exaggerated production of cytokines and impaired performance on cognitive tasks. Thus, developmental AE could interact with normal hormone production and baseline differences in microglial colonization of the brain to create sex-specific changes in neuroimmune activation and signaling with long-lasting consequences on brain function and behavior.

The current study found significant sex differences in microglial cell number and pro-inflammatory cytokine expression in the hippocampus during early postnatal development. A single-day binge-like alcohol exposure during the third trimester-equivalent (PD4) alters these measures in a region, timing, and sex-specific manner. This study also supports previous work that microglial colonization in the hippocampus occurs in a sex- and region-specific manner (Schwarz et al. 2012), with male neonatal pups having more microglia during the early postnatal period while females add a significant number of microglia over the first week. In summary, the current study supports that a single day binge of alcohol during the third trimester-equivalent is sufficient to activate the neuroimmune system though the response profile depends on a number of factors, including sex, brain region, and timing of analysis. More work is needed to determine if the observed alcohol effects persist into adulthood or if the immune system has been primed to exhibit an exaggerated or improperly regulated response following another immune or stress challenges later in life. Given that cognitive, behavioral, and social deficits are commonly found in individuals with prenatal alcohol exposure, it is plausible that aberrant immune system activation and long-term changes to neuroimmune function could contribute to the presence and severity of detrimental effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Representative images of microglia in the developing rat hippocampus. A) Image of the PD5 rat hippocampus labeled with the microglia-specific antibody Iba-1. Examples of subregions used for microglial cell count estimates are indicated. Scale bar = 200 μ m. B) Microglia found in CA1 of the female PD8 hippocampus (10 $\times$ lens). Scale bar = 100 μ m

Fig. 2.

Average Weights (in grams) of male and female rat pups combined, across neonatal conditions on postnatal days 4, 5, and 8. SC = suckle control, SI = sham-intubated, AE = alcohol-exposed. Blood Alcohol Concentrations (BAC) are expressed as milligrams per deciliter (mg/dl) $\pm$ SEM

Fig. 3.

Estimated number of Iba-1+ microglia in the hippocampus of PD5 male and female rat pups following PD4 alcohol exposure. Cell counts were conducted in the following the hippocampal subregions: **a** dentate gyrus molecular layer, **b** granule cell layer (GCL), and **c** hilus; **d** CA1 stratum oriens, **e** pyramidal cell layer (PCL), and **f** stratum radiatum; **g** CA3 stratum oriens, **h** PCL, and **i** stratum radiatum. Main effects of sex are indicated with lines/asterisks, while significant post hoc tests are indicated with letters. * = $p < 0.05$, ** = $p < .01$, *** = $p < 0.001$, **** = $p < 0.0001$. b: Male AE vs. Female AE, c: Male AE vs. Female Con; d: Male Con vs. Female AE. SC = suckle control, SI = sham-intubated, AE = alcohol-exposed; $n = 5-7/\text{group}$ (AE) or $10-15/\text{group}$ (controls). All data are expressed as mean $\pm$ SEM

Fig. 4.

Estimated number of Iba-1+ microglia in PD8 male and female rat pups following PD4 alcohol exposure. Cell counts were conducted in the following the hippocampal subregions: **a** dentate gyrus molecular layer, **b** granule cell layer (GCL), and **c** hilus; **d** CA1 stratum oriens, **e** pyramidal cell layer (PCL), and **f** stratum radiatum; **g** CA3 stratum oriens, **h** PCL, and **i** stratum radiatum. Significant post hoc tests are indicated with letters. e: Male Con vs. Female Con, f: Female AE vs. Female Con. SC = suckle control, SI = sham-intubated, AE = alcohol-exposed; n = 5–7/group (AE) or 10–15/group (controls). All data are expressed as mean $\pm$ SEM

Fig. 5.

Cytokine and chemokine gene expression in the hippocampus on (a) PD5 and (b) PD8 in male and female rat pups following PD4 alcohol exposure, as measured by RT-PCR. Main effects of treatment are indicated with bracketed lines and main effects of sex are indicated with unbracketed lines. Significant post hoc tests are indicated with letters. * = $p < 0.05$. b: Male AE vs. Female AE, d: Male Con vs. Female AE, f: Female AE vs. Female Con. SC = suckle control, SI = sham-intubated, AE = alcohol-exposed; $n = 6-8/\text{group}$ (AE) or $11-13/\text{group}$ (controls). All data are expressed as fold change compared to the control group (expressed as 1 on the graph) $\pm$ SEM

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Table 1

Number of animals used for each time point separated by assay and sex

Assay	Treatment	PD5		PD8	
		Males	Females	Males	Females
Cell counts	SC	8	9	6	5
	SI	5	6	5	5
	AE	6	7	5	5
Gene expression	SC	6	7	6	5
	SI	5	6	7	6
	AE	8	6	6	6

Table 2

List of gene expression primers

Gene	Encoded protein	Life Technologies cat. #
<i>Il1b</i>	Interleukin-1 β (IL-1 β)	Rn00580432
<i>Ccl4</i>	Chemokine (C-C motif) ligand 4 (CCL4)	Rn00671924
<i>Tnf</i>	Tumor necrosis factor- α (TNF- α)	Rn01525859
<i>Itgam</i>	Cluster of differentiation molecule 11B (CD11b)	Rn00709342
<i>Tgfb1</i>	Transforming growth factor- β (TGF- β)	Rn00572010
<i>Tubulin</i>	Tubulin	Rn01435337

Table 3

Statistics

Analysis	Group Comparisons	F	df	p-value	Post hoc
<i>Weights</i>	Repeated- measures	PD4 vs. PD5	789.5	1, 63	****
					N/A
	Interaction: Day x Treatment	PD4 vs. PD5	3.25	2, 63	***
					n.s.
<i>Cell Counts: PD5</i>	Repeated- measures	PD4 vs. PD8	3539.4	1, 58	****
					N/A
	DG: Molecular layer		20.58	1, 36	****
					N/A
	DG: GCL		10.36	1, 37	**
					N/A
	CA1: Stratum Oriens		8.017	1, 36	**
					N/A
	CA1: PCL		14.69	1, 37	***
					N/A
<i>Cell Counts: PD8</i>	CA1: Stratum radiatum		8.758	1, 37	**
					N/A
	CA3: Stratum Oriens		7.829	1, 37	**
					N/A
	CA3: PCL		7.829	1, 37	*
					N/A
	CA3: Stratum radiatum		7.246	1, 36	*
					N/A
	DG: Molecular layer		6.41	1, 36	*
					Male AE vs. Fem Con: *** Male AE vs. Female AE: ** Male Con vs. Fem AE: ***
<i>Cell Counts Across Days</i>	Interaction: Sex x Treatment	DG: GCL	6.058	1, 37	*
					Male AE vs. Fem Con: *** Male AE vs. Female AE: **
	DG: Hilus		4.875	1, 37	*
					n.s.
	DG: Molecular layer		5.20	1, 27	*
					n.s.
	DG: GCL		6.698	1, 27	*
					Fem AE vs. Fem Con: *
	Interaction: Sex x Treatment	CA1: PCL	4.58	1, 27	*
					n.s.
<i>Cell Counts: PD8</i>	CA3: Stratum Oriens		9.932	1, 26	**
					n.s.
	CA3: PCL		5.252	1, 27	*
					Fem AE vs. Fem Con: ** Male Con vs. Fem Con: **
	CA3: Stratum radiatum		4.571	1, 27	*
					Male Con vs. Fem Con: *
	DG: Molecular layer		21.74	1, 32	****
					N/A
	DG: GCL		16.14	1, 37	***
					N/A
<i>Cell Counts Across Days</i>	DG: Hilus		29.21	1, 36	****
					N/A
	CA1: Stratum Oriens		12.21	1, 36	***
					N/A

Analysis	Group Comparisons			F	df	p-value	Post hoc
mRNA: PD5	Females: PD5 vs. PD8	CA1: PCL	7.946	1, 37	***	N/A	
		CA1: Stratum radiatum	19.23	1, 37	****	N/A	
		CA3: Stratum Oriens	30.9	1, 36	****	N/A	
		CA3: PCL	15.97	1, 37	***	N/A	
		CA3: Stratum radiatum	30.9	1, 36	****	N/A	
		DG: Molecular layer	13.44	1, 32	***	N/A	
	Females: AE vs. Con	DG: GCL	8.138	1, 37	**	N/A	
		DG: Hilus	8.005	1, 37	**	N/A	
	Males: PD5 vs. PD8	DG: Hilus	24.65	1, 31	****	N/A	
		IL-1β	6.145	1, 34	*	N/A	
mRNA: PD5	AE vs. Con	CCL4	5.833	1, 32	*	N/A	
		CCL4	5.717	1, 32	*	N/A	
	Male vs. Female	CD11b	6.251	1, 24	*	N/A	
	Interaction: Sex x Treatment	CCL	4.613	1, 32	*	Fem AE vs. Male AE: * Fem AE vs. Fem Con: *	
	AE vs. Con	CCL4	5.556	1, 30	*	N/A	
mRNA: PD8		CCL4	10.49	1, 30	**	N/A	
	Male vs. Female	TNF-α	4.564	1, 27	*	N/A	
	Interaction: Sex x Treatment	CCL4	11.8	1, 30	**	Fem AE vs. Male AE: ** Fem AE vs. Fem Con: *	
		TNF-α	6.263	1, 27	*	Fem AE vs. Male AE: ** Fem AE vs. Fem Con: *	