MICROGLIAL ACTIVATION IN THE DEVELOPING RODENT BRAIN FOLLOWING BINGE-LIKE ALCOHOL EXPOSURE DURING THE THIRD TRIMESTER-EQUIVALENT

by

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ABSTRACT

Previous studies using rat models have looked at microglial activation following alcohol exposure in the adolescent and adult brains, or the long-term effects of neonatal alcohol exposure. This current study fills in a gap in the literature by observing the short-term immune response of the brain to neonatal alcohol exposure by measuring microglial cell counts, morphological activation, and inflammatory cytokine expression. Animals were exposed to alcohol on postnatal days (PD) 4-9 to model binge-like exposure during the third trimester equivalent in humans, and effects were observed on PD10. We hypothesized that neonatal alcohol exposure would lead to an increase in microglial proliferation, microglial activation (based on morphological status, or appearance), and inflammatory cytokine release, as these effects have been observed in the adolescent and adult brain following alcohol exposure. The results here were mixed, both supporting this hypothesis and contradicting it. A decrease in microglial cell counts was seen in alcohol-exposed (AE) and sham-intubated (SI) animals, possibly indicating an effect of stress on microglial cell number. No significant change in morphological status was found; however, we did see an increase in the pro-inflammatory cytokine IL-1β and the microglial activation marker, CD11b. These findings are significant as they show specific effects of alcohol on microglia in developing brain and possible stress effects on microglial activation.

Chapter 1

INTRODUCTION

1.1 Fetal Alcohol Spectrum Disorders

Alcohol use during pregnancy can lead to the development of Fetal Alcohol Spectrum Disorders (FASDs), which are estimated to affect 1-5% of live births each year (Sampson et al., 1997). FASD is an umbrella term that encompasses a variety of disorders, with the most severe being Fetal Alcohol Syndrome (FAS), which is characterized by facial dysmorphologies, growth and cognitive deficiencies, and long-lasting damage to the central nervous system (CNS). CDC studies have estimated the prevalence of FAS to be 0.2 to 1.5 infants per 1,000 live births; the most recent study found 0.3 infants with FAS per 1,000 live births in children ages 7-9 (CDC, 2002, 2015). FASDs also include Alcohol-Related Neurodevelopmental Disorders (ARND) which involve deficits in CNS function without craniofacial abnormalities, and Alcohol-Related Birth Defects (ARBD) which involve problems with internal organ function, including the heart, kidneys, and bones, as well as hearing loss (CDC, 2015). It is estimated that the lifetime cost for one individual with FAS is $2 million, with an annual cost of over $4 billion cumulatively in the United States (Lupton et al., 2004).

Neurocognitive and behavioral deficits in IQ, executive functioning, learning and memory, language expression and comprehension, attention, and social functioning due to FASDs can persist into adulthood and affect the individual throughout their lifetime (Mattson & Riley, 1998). Children with FASD exhibit significantly smaller brain volumes, which in rat models, has been linked to both

reduced survival of proliferated cells and increased apoptosis of post-mitotic neurons (Miller, 1998, Ikonomidou et al., 2000).

The brain is particularly vulnerable to the effects of alcohol during a period known as the "brain growth spurt" which occurs during the third trimester of fetal development in humans, or the equivalent first two postnatal weeks in rodents. When ethanol is administered in a binge-like manner to achieve high blood alcohol concentrations (BACs) during this period, significant apoptosis and cell loss can be observed in several brain areas including the cerebral cortex, cerebellum, and the hippocampus (Goodlet and Eilers, 1997, Klintsova et al., 2007, Mooney et al., 1996).

Rodent models of FASD have shown significant impairments in spatial learning and memory (Berman and Hannigan, 2000, Johnson and Goodlett, 2002) through damage to hippocampal plasticity mechanisms, such as adult neurogenesis, long-term potentiation (LTP) and dendritic complexity (Anderson et al., 2012, Hamilton et al., 2010, Lovinger et al., 1989). Specifically, the aforementioned studies have shown alcohol-induced decreases in cell proliferation, NMDA receptor activation, and dendritic spine density in adult rats. Disruption of these cellular mechanisms are correlated with the cognitive and behavioral deficits seen in rodent models of FASD.

1.2 Various Roles of Microglia in the CNS

Microglia are the resident immune cells of the CNS and serve a diverse array of functions within the brain, especially during neurodevelopmental processes. These mononuclear phagocytes are the primary responders to immune challenges and will exhibit various activation states based on the current homeostatic needs of the CNS (Nimmerjahn et al., 2005). Microglia can thus serve both a neurodegenerative role

(classical or M1 macrophage activation) or a neuroprotective role (alternative or M2 activation). Microglia can exist in either an active or inactivated state, with each displaying a distinct morphological appearance, as well as differential release of pro-inflammatory (M1) or anti-inflammatory (M2) cytokines and chemokines. A resting, quiescent state is characterized by a small soma and long thin processes for surveying nearby tissue for insults and injury. A partial activation state is characterized by an enlarged soma, and shortened, thicker processes. And finally, a fully-activated or phagocytic state is characterized by a round shape with even short processes, or an amoeboid shape (Olah et al., 2011). These activation states are depicted in Figure 1, C, D, and E, respectively. Microglia act as antigen-presenting cells (APCs), and upon activation will engulf and phagocytose damaged cells and cellular debris (Aloisi, 2001).

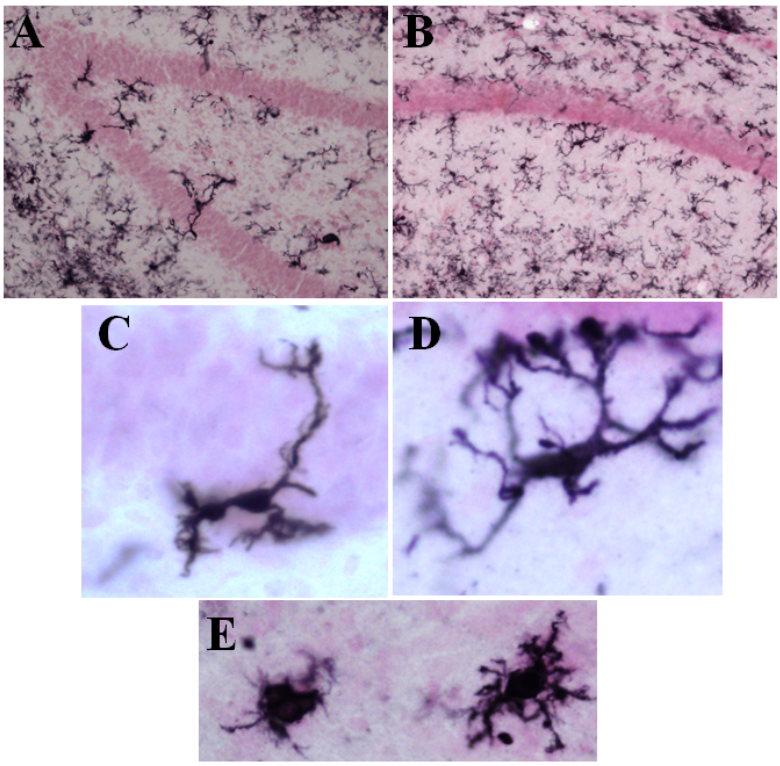

Figure **1.** – **A)** Various microglial activation states in dentate gyrus (DG) of PD10 hippocampal tissue. Image taken with 20X objective.**B)** CA1 (20X) **C)** Ramified phenotype (40X) **D)** Partial-Activation phenotype **E)** Fully-Activated phenotypes

Microglia originate from macrophage precursors in the peripheral nervous system (PNS), more specifically, myeloid cells in the bone marrow, and begin migrating to and colonizing the brain during embryonic days (ED) 9-10 (Chan, Kohsaka, and Rezaie, 2007, Ginhoux et al., 2010). Amoeboid microglia heavily colonize the developing hippocampus in rats by (ED17) and by birth, PD0, begin to develop short, thick branches which then protract even further by PD4. Finally, by

young adulthood, PD30, microglia exhibit the ramified morphology that persists into adulthood (Schwarz and Bilbo, 2014, 296).

In addition to phagocytosing damaged cells and cellular debris, microglia exhibit several other important roles in neurodevelopment in addition to maintaining homeostasis of the CNS such as synaptic pruning of neurons (Paolicelli et al., 2011). Microglia maintain tissue homeostasis through neurotrophic support of remyelination of damaged areas, during and following the onset of demyelination (Olah et al., 2012). Sierra and colleagues (2010) found that microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis, indicating a homeostatic role for microglia in the neurogenic cascade. These studies show distinct, new roles of microglia in maintaining the integrity of the CNS and that they are crucial cells in a variety of functions. Disruption of these functions through aberrant activation of microglia can, however, have persistent, detrimental effects on the brain.

While the immune system plays an important role in the developing brain, early-life immune activation has been shown to have long-term consequences. Aberrant activation increases persistent levels of pro-inflammatory cytokines in the brain that can later lead to neurodevelopmental and psychopathological disorders like schizophrenia and depression (Cai et al., 2000, Meyer et al., 2006, Urakubo et al., 2001).

1.3 Ethanol-Induced Activation of Microglia

Ethanol exposure can induce death and structural change of neurons, but more recent studies have shown that microglia may be even more sensitive to the effects of alcohol. Increased activation of microglia and levels of associated cytokines have been

strongly linked with ethanol-induced neuroinflammation, and are recognized at least as a consequence of insult if not the actual source of damage (Marshall et al., 2013). Elevated glutamate levels have also been seen following binge-alcohol exposure which lead to phagocytic activation, release of inflammatory cytokines, and corresponding neuroinflammation (Block et al., 2007, Ward et al., 2009). Whether the activation of microglia is the cause or consequence of neuroinflammation remains unclear. Neuroinflammation is a necessary response to brain trauma or pathogenic intrusion, but sustained activation of microglia can inflate levels of cytotoxicity through higher levels of inflammatory cytokines causing neuronal injury rather than preservation. Following acute or subchronic exposure, microglia may initially exhibit a neuroprotective role through their release of anti-inflammatory cytokines, but may then shift to release pro-inflammatory cytokines resulting in neurotoxic effects (Yang et al., 2014).

Four-day binge-alcohol exposure in adult animals has been shown to trigger a partial activation of microglia, but not a full activation (McClain et al., 2011, Marshall et al., 2013). It is likely that further exposure will induce full, phagocytic activation and increased apoptosis of neurons (McClain et al. 2011). Based on these studies, it is possible that exposure to alcohol during development may induce activation of microglia that persists into adulthood. The initial alcohol exposure could serve to prime microglia to future insults, such as an immune challenge, stress, or further alcohol exposure. Primed microglia would shift to an activated state more quickly than those in a quiescent state, leading to an exaggerated immune response that could have neurotoxic effects.

1.4 Rationale for Current Research

Past experiments have studied microglial activation following alcohol exposure on the adolescent brain and the adult brain, but gaps in the literature remain regarding the effects of neonatal alcohol exposure on microglial activation. Tiwari and Chopra (2011) showed that ethanol exposure on PD7-9 resulted in increased pro-inflammatory cytokines compared to controls when observed three weeks later on PD28. McClain and colleagues (2011) showed that four-day binge ethanol exposure during adolescence triggers a morphological shift in resting microglia to partial activation and increases microglial proliferation throughout the hippocampus. The initial exposure to the alcohol may have served to prime the microglia to future insults as they retained their morphological characteristics into adulthood. Marshall and colleagues (2013) observed that binge-alcohol exposure in adult rats also induced a partial activation of microglia, as well as increased proliferation and migration to damage sites. Potential priming of the microglia was demonstrated in this study as well by the lasting presence of markers of activation such as morphology and cytokine levels, indicating that continued alcohol exposure could lead to an exaggerated immune response resulting in neurodegeneration.

The goal of the current study is to investigate whether third trimester-equivalent binge-like alcohol exposure affects short-term microglial activation in the neonatal brain through analysis of total microglial cell number, microglial activation state, and pro-inflammatory cytokine gene expression. It is hypothesized that alcohol exposure will lead to increased proliferation and activation of microglia, as has been seen in later time points in previous work, and an increase in gene expression of pro-inflammatory cytokines (McClain et al., 2011, Marshall et al., 2013). An increased immune response of microglia to alcohol at this time point would be likely to correlate

with neurodegeneration seen by alcohol-induced apoptosis, and persistent activation with the long-lasting cognitive deficits.

Chapter 2

METHODS

2.1 Animals

Timed-pregnant Long-Evans rat dams were acquired from Harlan Laboratories (Indianapolis, IN) and housed in cages of standard dimensions (17 cm high x 145 cm long x 24 cm wide) in a 12/12 hr light cycle (lights on at 9:00 AM) upon arrival. On postnatal day (PD) 3, each litter was culled to eight pups (6 male, 2 female 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 PD10. Only male pups were used for analyses for this current project, for a total of 67 animals. Future work will examine data from both sexes. All procedures were carried out in accordance with the animal use protocol approved by University of Delaware Institutional Animal Care and Use Committee.

2.2 Binge-Alcohol Model

AE pups received ethanol in a milk formula through intragastric intubation according to a model for binge-exposure (2 feedings, 2 hours apart, total dose 5.25 g/kg/day) on PD 4-9. Specifically, on the first day of AE, rats received 2 alcohol and 2 milk feedings, while on the following days they received 2 alcohol and only1 milk feedings as they become accustomed to AE and can suckle from the dams again. Rats

in the SI condition were intubated as well but received no liquid solution to control for the possible stress effects caused by the intubation procedure. SC pups remained undisturbed with the dam except for daily weight measurements to insure proper, healthy development. On PD10, the rats were sacrificed by transcardial perfusion or rapid decapitation (for tissue sectioning or homogenization, respectively) so that the short-term effects of alcohol on the developing brain could be assessed.

2.3 Blood-Alcohol Concentrations (BACs)

On PD4, 90 minutes post-second-intubation, blood samples were collected through tail clippings from both the AE and SI groups to determine the blood alcohol concentrations. Blood was only 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/15-25 minutes) and the plasma collected and stored at -20ºC until analysis. Plasma was analyzed for BAC using an Analox GL5 Alcohol Analyzer (Analox Instruments, Boston, MA).

2.4 Immunohistochemistry

Animals were perfused with heparinized 0.1M phosphate-buffered saline (PBS; pH 7.2) followed by 4% paraformaldehyde in PBS (pH 7.2). Brains were stored in 4% paraformaldehyde for 2 days, then transferred to 30% sucrose in 4% paraformaldehyde for approximately one week until sectioning. Brains were sectioned at 40 μm on a cryostat at -20°C, throughout the entire hippocampus and stored at 20°C in cryoprotectant solution. A systematic random sampling procedure, every 12th section, was used in selecting the sections for processing.

Microglial cells were labeled with microglial-specific marker Ionized calcium-Binding Adaptor molecule 1 (Iba-1). Sections were washed three times in 50mM tris-buffer solution (TBS) and then incubated in 2% H_2O_2 in 70% methanol for ten minutes to eliminate endogenous peroxidases. Non-specific binding sites were blocked by incubation for 1 hour in 3% normal goat serum (Sigma Life Sciences) + 1% Triton-X (LabChem Inc) in TBS. Then sections were transferred into primary antibody anti-Iba-1 made in rabbit (1:5000; Wako Chemicals) in blocking solution, and incubated for 48 hours at 4°C. Sections were then washed three times in 50mM TBS to rinse off the primary antibody before entering incubation in secondary antibody (anti-rabbit IgG made in goat, 1:200, Sigma) for 1 hour at room temperature in washing solution (3% goat serum in 50mM TBS). The tissue was then rinsed 2 times in TBS and one in washing solution before being transferred to ABC solution (Vector Laboratories, Burlingame, CA) for 1 hour to amplify the signal. Finally, localization of binding sites were visualized by reaction between biotinylated HRP (in ABC) and nickel-enhanced diaminobenzidine (DAB).

2.5 Stereology

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, using the optical fractionator probe (Stereo Investigator, MBF Bioscience., Williston, VT). The StereoInvestigator software calculates the total volume of the outlined brain region taking into consideration the 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 on each section. For cell number quantification, the following parameters were used: sampling grid frame of 200 X 200 micrometers, counting frame of 100 X 100

micrometers, a guard zone of 2 micrometers, and a dissector height of 12 micrometers. For cell morphology assessments, the parameters were as follows: sampling grid frame of 400 X 400 micrometers, counting frame of 100 X 100 micrometers, a guard zone of 2 micrometers, and a dissector height of 12 micrometers. Section thickness was measured at every 5th counting site. An average section thickness was computed by the software and used to estimate the total volume of the sample region and the total number of Iba-1+ cells (thickness sampling fraction, tsf = # micrometers/ section thickness). The mean coefficient of error (CE) for the number of cells did not exceed the recommended m = 0.1. The total number of cells was estimated using the following formula:

$$N = \sum Q^{-} \cdot \frac{t}{h} \cdot \frac{1}{asf} \cdot \frac{1}{ssf}$$

After completion of cell counts, morphologies of the microglia were independently assessed for 27 animals to investigate activation from alcohol exposure and to compare across hippocampal regions. Inactive microglia were assigned the label “1,” partially-activated microglia “2,” and fully-activated microglia “3” for discrimination during analysis.

2.6 Polymerase Chain Reaction (PCR)

Following rapid decapitation on PD10, the hippocampus was dissected on dry ice and mounted onto slides which were stored at -80°C until homogenization. Tissue was homogenized in lysis buffer RLT Plus (Qiagen). DNA/RNA were extracted using the AllPrep DNA/RNA Mini Kit (Qiagen Inc., Valencia, Calif., USA). RNA concentrations were analyzed using spectrophotometry (NanoDrop 2000,

ThermoScientific), after which RNA was stored at -80°C. cDNA was synthesized from the RNA using the Quantitect Reverse Transcription kit (Qiagen). Gene expression was assessed for a total of 36 animals using real-time PCR (Bio-Rad CFX96) with SYBR probes (Bio-Rad) and forward and reverse primers for the *TNFA* gene for TNF-α, the *IL1B* gene for IL-1β, the *ITGAM* gene for CD11b, and the *CCL4* gene for CCL4. GAPDH was used as a reference gene. All reactions for each gene target and reference were run in duplicate. Product specificity was assessed by analysis of melt-curves for each sample. Fold change was measured per plate by comparing all samples to the sample with the smallest difference between the target gene and the reference gene.

2.7 Statistical Analysis

A repeated-measures analysis of variance (ANOVA) with the factors of day x neonatal treatment was performed for animal weights. For postnatal days 9 and 10, planned one-way ANOVAs were performed to compare weights within neonatal treatments on those days. For microglial cell counts and cytokine expression, one-way ANOVAs were performed to assess differences between neonatal treatments. When appropriate, *post hoc* Tukey's tests were run. A two-way ANOVA was performed for the analysis activation states with the factors of neonatal treatment x activation state. One-way ANOVAs were then performed to assess changes in activation state within each neonatal treatment. All data are expressed as mean ± standard error of the mean (SEM) except where otherwise noted. Statistical significance was set as $p < 0.05$. Trends of $p < 0.1$ are also mentioned when appropriate.

Chapter 3

RESULTS

3.1 Weights and blood alcohol concentrations

All animals were weighed daily on postnatal days 4-10. Table 1 shows the average weights for each condition at each date the animals were weighed. A main effect of day $F(2,63)=1252.6$, $p= 0.0001$, and a main effect of day by neonatal condition $F(4,128)=8.88$, $p= 0.0001$, on average weight was seen. Average BACs were obtained using blood samples taken after the first day of AE (PD4). The average BAC reached by AE animals was 328.7 mg/dl +/- 26.7 SEM. The outcome of this analysis demonstrates that (AE) animals had significantly lower weight by the end of treatment.

Table **1. -** Average Weights (in grams) across neonatal conditions on postnatal days 4, 9, and 10. SC = suckle control, SI = sham intubated, AE = alcohol exposed and Blood Alcohol Concentration (BAC) in milligrams per deciliter

	SC	**SI**	**AE**
PD4	10.93 ± 1.56	11.07 ± 0.66	10.87 ± 0.67
PD9	20.39 ± 2.85	20.71 ± 1.65	17.75 ± 1.85
PD10	22.55 ± 2.89	22.83 ± 1.89	19.57 ± 2.43
BAC			328.7 ± 26.7

3.2 Microglial Cell Counts

Microglial cell counts were determined using unbiased stereological analysis of Iba-1+ cells in the hippocampus. Region-specific effects were observed in the DG and CA1 regions. A one-way ANOVA found a main effect of treatment on Iba-1+ cells counts in the DG ($F(2,18)=25.56$, $p= 0.0001$), and in the CA1 region ($F(2,18)=5.433$, $p=.0143$) was found. Within these regions, a significant decrease of cells was seen in the SI and AE groups compared to the SC group (Tukey's post hoc: $p < 0.05$). A significant difference between SI and AE was also found ($p = 0.005$), with AE group having a higher cell count compared to SI. There were no significant effects of treatment on cell counts seen in the CA3 region.

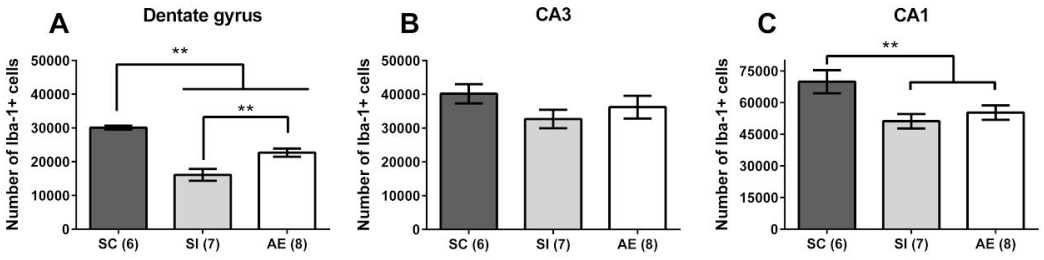

Figure **2. -** Iba-1+ cell counts. **A)** dentate gyrus, **B)** CA3, **C)** CA1 ** = $p < 0.01$, SC = suckle control, SI = sham intubated, AE = alcohol exposed All data are expressed as mean ± standard error of the mean (SEM).

3.3 Microglial Morphologies

Microglial morphologies were analyzed to assess activation states in each hippocampal subregion. For all regions and neonatal conditions, approximately 40% of cells exhibited a resting state, 40% a partial activation state, and 20% a full activation state. For dentate gyrus, a main effect of activation state was found

($F(2,51)=46.65$, $p < 0.0001$; Figure 3A). For CA3, a main effect of activation state was found ($F(2.52=53.84$, $p < 0.0001$; Figure 3B). A trending activation state x neonatal condition interaction was also found in CA3 ($F(4,52)=2.449$, $p = 0.0576$). For CA1, a main effect of activation state was found ($F(2,51)=34.20$, $p < 0.0001$; Figure 3C). No main effect of neonatal treatment or interaction was found in DG or CA1 ($p > 0.05$). *Post hoc* analysis of activation state (Tukey's multiple comparisons test) found a significant difference between resting and partial activation compared to amoeboid activation in each region across all neonatal conditions with there being more resting and partially activated microglia compared with amoeboid (SC resting vs. amoeboid: $p < 0.01$; SC partial vs amoeboid $p < 0.01$; SI resting vs. amoeboid: $p <$ .01; SI partial vs amoeboid $p < 0.01$; AE resting vs. amoeboid: $p < 0.01$; AE partial vs amoeboid $p < 0.01$). No significant differences between percentage of resting versus partially activated microglia were found in any of the neonatal conditions (p = ns).

Amoeboid and partially-activated microglia groups were then combined into one group to compare total "resting" versus "active" microglia in each hippocampal subregion. For DG, a main effect of activation state was found ($F(1,34)=14.10$, $p = 0.0006$). For CA3, a main effect of activation state was found ($F(1,34)=7.406$, $p = 0.0102$). A significant interaction of activation state x neonatal condition was also found in CA3 ($F(2,34)=5.063$, $p = 0.0119$)For CA1, a main effect of activation state was found ($F(1,34)=31.66$, $p < 0.0001$). Amoeboid and partially-activated microglia groups were then combined into one group to compare total "resting" versus "active" microglia in each hippocampal subregion. For DG, a main effect of activation state was found ($F(1,34)=14.10$, $p = 0.0006$). For CA3, a main effect of activation state was found ($F(1,34)=7.406$, $p = 0.0102$). A significant interaction of activation state x

neonatal condition was also found in CA3 ($F(2,34)=5.063$, $p = 0.0119$)For CA1, a main effect of activation state was found ($F(1,34)=31.66$, $p < 0.0001$).

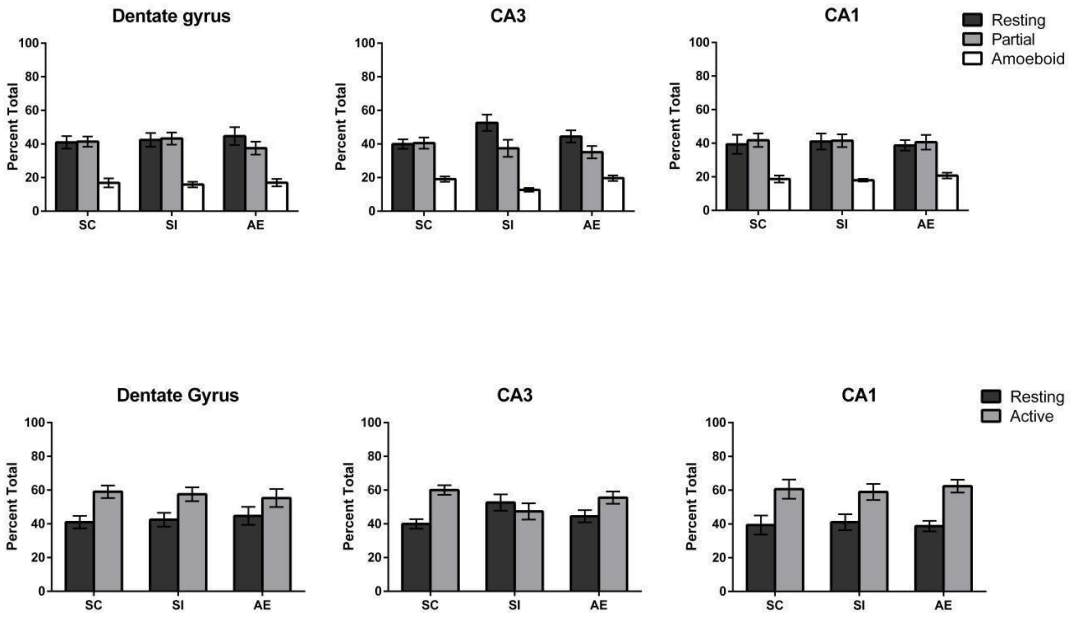

Figure **3. -** Morphological activation states of microglia in A) DG, **B)** CA3, **C)** CA1**.** Percentage of microglia in resting vs. activated states in D) DG, **E)** CA3 **F)** CA1. SC = suckle control, SI = sham intubated, AE = alcohol exposed. All data are expressed as mean ± standard error of the mean (SEM)

3.4 Pro-Inflammatory Cytokine Expression

Real-time quantitative Polymerase chain reaction (PCR) was performed to assess the levels of gene expression of the associated pro-inflammatory cytokines IL-1β, CD11b, TNF-α, and CCL4. A main effect of neonatal condition on IL-1β expression was found ($F(2,20)=3.966$, $p=0.0354$; Figure 4A), with significant increases of expression seen in the AE group compared to the SC group (Figure 4A;, *p*

< 0.05). A trending main effect was found for CD11b ($F(2,16)=2.596$, $p = 0.1056$; Figure 4B). However, no significant differences were seen across neonatal conditions for expression of TNF-α and CCL4 (p=ns).

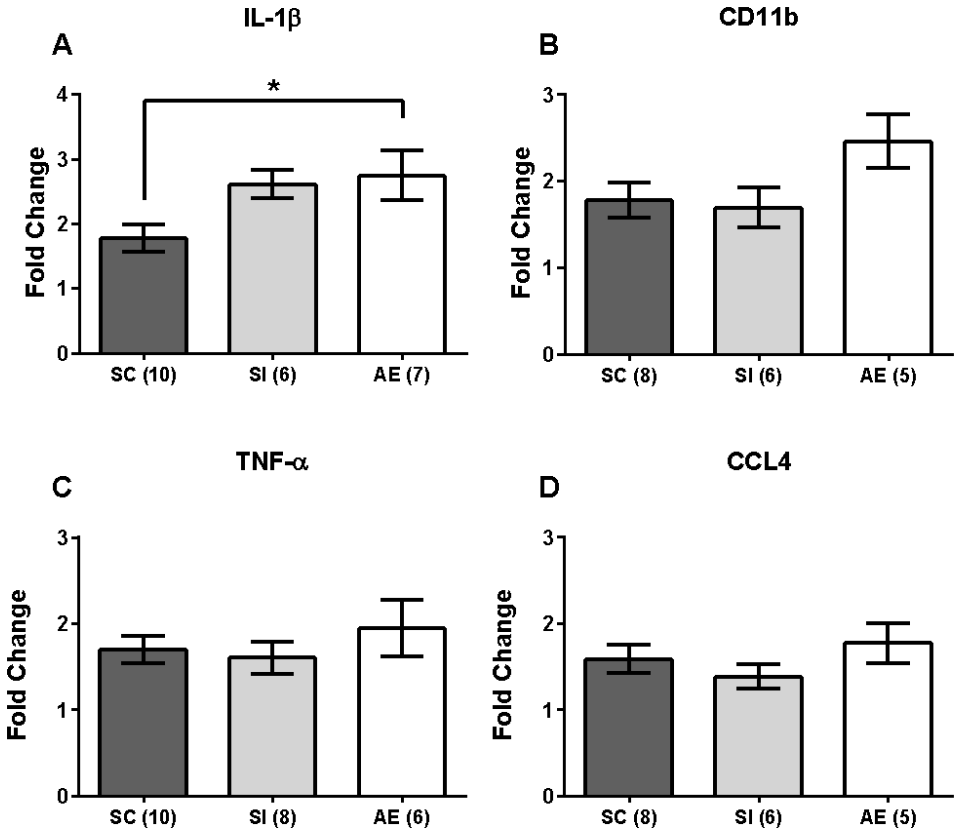

Figure **4. -** Gene expression of proinflammatory cytokines. **A)** IL-1β, **B)** CD11b, **C)** TNF-α, **D)** CCL4 * = $p < 0.05$, SC = suckle control, SI = sham intubated, AE = alcohol exposed. All data are expressed as mean ± standard error of the mean (SEM).

Chapter 4

DISCUSSION

4.1 Summary of Results

The current study set out to fill in a gap in the literature by observing the effects of third trimester-equivalent binge-like alcohol exposure on microglial response in the neonatal brain. Overall, we found reduced numbers of microglia in the alcohol-exposed and sham-intubated animals, no significant treatment-induced morphological changes, but increased levels of certain pro-inflammatory cytokines in sham-intubated and/or alcohol-exposed animals. Previous studies observed microglia only in the adult rodent brain tissue and found that ethanol exposure led to an increase in microglial proliferation and induced a partial activation of these cells (Marshall et al., 2013; McClain et al., 2011). The results of the current study show some significant effects of both neonatal alcohol and intubation stress in neonatal brain. However, these effects differ in many ways when compared to effects in the adult brain, implying that microglia may act on a different course while responding to alcohol administration in the neonatal brain. Additionally, our results highlight the profound effect that stress can have on the developing brain, as the sham-intubated animals also showed reduced microglial number and enhanced cytokine production.

4.2 Microglial Cell Counts

Region-specific differences in microglia numbers were seen in the DG and CA1 regions. In both of these regions there was a significant decrease in Iba-1+ cells in the AE and SI groups compared to the SC group. This result seemed counterintuitive when previous work has shown that high doses of alcohol exposure

can induce neuroinflammation and increase the proliferation of microglia in the adult brain (Ward et al., 2009, McClain et al., 2011). However, increased levels of corticosterone due to stress can act on glucocorticoid receptors on microglial cells to inhibit further proliferation (Ganter et al., 1992). Intragastric intubation on PD4-9 has been shown to elevate circulating levels of corticosterone in blood plasma of both AE and SI animals (Boschen et al., 2015). Thus, the stress of the intragastric intubation of the AE and SI groups in the present study could have induced a decrease in microglial proliferation compared to the controls, and then the alcohol exposure may have lessened this stress-affected decrease as alcohol has been shown to increase proliferation (McClain et al., 2011, Marshall et al., 2013). Stress may be playing a suppressive role while alcohol plays an activating role, possibly causing a conflicting interaction.

4.3 Morphological Activation of Microglia

No significant differences in morphological activation states of microglia were seen across neonatal treatment groups or hippocampal subregions (Figure 3). Approximately 40% of microglia were in a resting state, 40% in a partial activation state, and 20% in an amoeboid state. It is known that the brain is colonized by amoeboid microglia during development and shift to a resting state in adulthood (Giulian et al., 1988). A study from Schwarz, Sholar, and Bilbo (2013) investigated changes in morphological states across development for adolescent (PD30) and adult (PD60) animals. At PD30, there were approximately 46% in a resting state, 41% in a partial activation state, and 13% in an amoeboid state. At PD60, there were approximately 50% in a resting state, 37% in a partially activated state, and 13% in an amoeboid state. These data seem to show a natural shift towards the resting, ramified

phenotype as the animal ages. Studies of alcohol exposure to the adolescent and adult brain have shown a shift from microglia in a primarily resting state to a partial activation state (Marshall et al. 2013, McClain et al. 2011). This previous work suggests that on PD10 the microglia have not yet completed the shift in morphology from amoeboid to resting, so it is likely that differences in morphological activation following alcohol exposure are not seen due to naturally higher levels of partially- and fully-activated microglia at this neonatal time point.

4.4 Associated Cytokine Expression

The proinflammatory cytokines IL-1β, TNF-α and CCL4 and the microglial activation marker, CD11b, are all classical markers of microglial activation. Gene expression assays of these cytokines showed a significant increase of IL-1β expression in both the AE and SI groups compared to the SC group, as well as a significant increase between AE and SI groups. IL-1β is a mediator of inflammation and is involved in other cellular processes such as proliferation and apoptosis. The protease caspase-1 cleaves IL-1β from its inactive precursor form to its active, inflammatory form, and also mediates programmed cell death (Denes, 2012, Hogquist et al., 1991). No significant differences in expression of CD11b, TNF-α and CCL4 were seen across groups. It is possible that these particular cytokines are not involved in a response to alcohol exposure, and instead only lead to an inflammatory response following other unrelated insults, or just that expression is not significant due to low statistical power and differences may be seen when increasing the number of animals per group. These results correlate with studies of adult rats that specifically found no change in TNF-α expression following alcohol exposure (Marshall et al., 2010, McClain et al., 2011, Zahr et al., 2010).

Additionally, stress effects due to intragastric intubation are likely, as acute and chronic stress have been shown to suppress or increase release of cytokines (Elenkov and Chrousos, 2002). Beutler and colleagues (1986) showed that glucocorticoids may suppress inflammation by limiting production of inflammatory cytokines such as TNF-α. A study done by Elenkov and colleagues (1996) showed that glucocorticoids had no effect on anti-inflammatory cytokine IL-10, but that catecholamines actually increased its production. Another study showed that stress due to immobilization can elevate levels of IL-1β (Minami et al., 1991). Additionally, extremely small quantities of IL-1 in the brain can rapidly suppress immune responses and do so for prolonged periods of time (Weiss et al., 1989). For the results of this current study, this means that the observed increase of IL-1β expression cannot be completely attributed to alcohol as stress could be influencing its expression as well, as indicated by data from the SI group. The lack of an increase of TNF-α expression could be due to immune suppression by stress, or failure of alcohol to induce a full neuroinflammatory response. The possible interaction of alcohol and stress on cytokine levels needs to be further discerned with future studies.

4.5 Relevance to Previous Work

Our lab has previously reported that third trimester-equivalent alcohol exposure negative impacts long-term survival of newly generated neurons in the hippocampal dentate gyrus (Klintsova et al., 2007; Hamilton et al., 2012). In addition, wheel running exercise has been shown to enhance cell proliferation in control and alcohol exposed (AE) rats. Environmental complexity following wheel running was sufficient to rescue the number of surviving newly generated neurons and yield long-term survival rates in AE rats that were comparable to normally reared control rats.

These interventions provide a potential model for ameliorating hippocampal-dependent impairments associated with FASDs. The activation of microglia in response to alcohol exposure is likely involved in these previously reported findings. Microglia may be contributing to neuroinflammation leading to the degeneration of neurons, and could also be contributing to anti-inflammatory, neuroprotective, and homeostatic responses with WR and EC. Previous work in our lab showed increases in BDNF protein levels and mRNA expression in the hippocampus, suggesting a potential neuroprotective mechanism following alcohol exposure, and it is known that microglia can release BDNF upon activation (Coull et al., 2005, Gomes et al., 2013).

4.6 Relevance to FASD in humans

Further understanding of the brain's immune response to alcohol exposure in rodent models will give insight to analogous responses in humans with FASD. Though not all mechanisms and dynamics of the rodent CNS immune system are likely to apply to humans, it is reasonable to expect that the major innate responses are similar in effect. Studies of alcohol abuse in humans have also shown higher blood-plasma levels of inflammatory cytokines such as IL-1β with long-term abuse (Achur et al., 2010), and higher levels of anti-inflammatory cytokines such as IL-10 following a just a single binge (Kim et al., 2003). Understanding the effects of alcohol on microglial activation and function could lead to treatments for FASD in the future to ameliorate deficits due to neuroinflammation and degeneration.

4.7 Conclusions and Future Directions

The present study sought to find the short-term effects of ethanol-exposure on microglial activation in the neonatal brain. It was expected that alcohol would lead to

increased proliferation of microglia throughout the hippocampus, as well as lead to increased morphological activation; both of these effects seen in older rats in studies with similar binge-alcohol models (Marshall et al., 2013, McClain et al., 2011). However, the results were unexpectedly the opposite of this hypothesis: significant decreases in microglial cell counts were found in the hippocampus of AE animals (as well as in SI animals) compared to controls, and no significant differences in morphological activation were observed between experimental conditions. These unpredicted outcomes in microglial activation following alcohol exposure to the developing brain compared to the adult brain highlight the need for further studies.

One future direction for this line of study would be to observe microglial proliferation, morphology, and cytokine expression following a single neonatal alcohol exposure, which should reduce the effect of stress on the animal and allow a better observation of alcohol effects. Long-term effects following developmental alcohol exposure and the effects of future immune insults on microglia should be assessed as well to see if priming in activation can exacerbate the response. Additionally, sex differences in microglial and cytokine response are a critical next avenue of research, as work has shown differentially activated microglia in the naturally developing brain (Lenz et al., 2013). The current study focused on pro-inflammatory cytokine gene expression to assess the potential for neurodegenerative damage caused by this model of alcohol exposure. Future work should also measure anti-inflammatory cytokine expression to assess a possible neuroprotective response of microglia, as increases in neurotrophic factors like BDNF have been shown with this model of AE by our lab at this current time-point (Boschen et al., 2015). In this

manner, a timeline of microglial activity can be established and the pathophysiology of alcohol exposure in the developing brain can be further understood.

In summary, data from this study suggest distinct differences in alcohol's effects on microglial activation in the developing brain verses those in the mature brain. While these studies add valuable information to the FASD literature, much of the brain's immediate and long-term immune response following teratogenic insult during this crucial and delicate period of brain development remains to be discovered.

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