MICROGLIAL ACTIVATION IN THE DEVELOPING RODENT BRAIN FOLLOWING SINGLE-DAY MODERATE BINGE-ALCOHOL EXPOSURE

by

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ABSTRACT

Alcohol has been shown to activate microglia, the immune cells of the brain, which may contribute to lasting deficits in cognitive functioning seen in humans with Fetal Alcohol Spectrum Disorders (FASD) and in rodent models of FASD. 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 adds to the newly growing literature on the short-term immune response of the brain to neonatal alcohol exposure, by expanding the timeline of microglial activation through measurement of microglial cell counts and inflammatory cytokine expression in the developing hippocampus. Cells were counted in three separate subregions of the three hippocampal subfields: Dentate gyrus: hilus, granule cell layer, molecular layer; CA1 and CA3: stratum oriens, pyramidal cell layer, stratum radiatum. Male and female rat pups were exposed to alcohol on postnatal day (PD) 4 to model a single binge-like exposure during the third trimester in humans, and effects within the hippocampus were measured on PD5 and PD8. We hypothesized that neonatal alcohol exposure would lead to an increase in microglial cell counts and inflammatory cytokine release, and that sex differences would be observed. The results support this hypothesis: an increase in PD5 microglial cell counts was seen in alcohol-exposed (AE) male rats in the hilus and molecular layer of the dentate gyrus compared to suckle controls (SC). Males generally had higher levels

of microglia in all hippocampal subregions except for the hilus of the dentate gyrus, when compared with females. Females exhibited an opposite effect in the dentate gyrus: a decrease in cell counts following alcohol exposure in the granule cell layer and molecular layer when compared to sham-intubated (SI) controls. The increases in the pro-inflammatory cytokines CCL4 and IL-1β in alcohol exposed animals were not statistically significant compared to controls, but may reach significance upon further addition of animals. However, when the data from both sexes were combined, significant increases in CCL4 expression were seen in AE and SI animals compared to SC. following These findings are significant as they add to our knowledge of specific sex-dependent effects of alcohol on microglia in developing brain.

Chapter 1

INTRODUCTION

1.1 Fetal Alcohol Spectrum Disorders

Alcohol intake during pregnancy can lead to the development of Fetal Alcohol Spectrum Disorders (FASD), the leading cause of birth defects and mental retardation in the United States despite being entirely preventable (Bailey & Sokol 2008; Stratton et al., 1996). FASD is an umbrella term that encompasses a variety of disorders that affect the exposed individual throughout their lifetime. Fetal alcohol syndrome (FAS) is the most severe form of these disorders and is characterized by craniofacial dysmorphologies such as a smooth philtrum, thin upper lip, and epicanthal folds, persistent damage to the central nervous system, and growth and cognitive deficiencies. Although an accurate and discrete number is difficult to isolate, the Center for Disease Control (CDC) reports an estimated prevalence of 0.2 to 1.5 cases of FAS per 1,000 live births in the United States (CDC, 2015). This rate of occurrence has been found to be higher, however, in some countries of the world. For example, the NIH estimates the prevalence of FASDs to be as high as 60 cases per 1,000 individuals in South Africa. Additionally, it is estimated that for each child diagnosed with FAS, there are three children with other forms of FASDs without the physical signs of FAS (NIH, 2013). FASDs also include: Alcohol-Related Neurodevelopmental Disorders (ARND) which involve deficits in central nervous system (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). Unfortunately, these completely preventable disorders cost the United States approximately $2 million per lifetime of an affected individual, exceeding $4 billion cumulatively (Lupton et al., 2004).

Typically, children with FASDs exhibit deficits in executive functioning, including attention, spatial memory, and planning and strategy use (Green et al., 2009). It has long been shown that alcohol use during pregnancy causes lower IQs in offspring (Streissguth et al, 1989, 1990; Mattson & Riley, 1998), but a more recent study sought to tease out which neurocognitive and behavioral deficits are due more to alcohol specifically than to resulting lower IQs. Children with heavy prenatal alcohol exposure were matched with controls with similar IQs, and it was found that those exposed to alcohol exhibited significantly greater deficits in verbal learning and expression of pathological behavior compared to controls (Vaurio et al., 2011). These cognitive and social deficits correlate with volumetric analyses of brains of children with FASDs, which show cortical thinning and reductions in total brain volumes and weights (Zhao et al., 2011; Lebel et al., 2008).

Several brain structures, such as the hippocampus, cerebellum, and prefrontal cortex, are particularly sensitive to the teratogenic effects of alcohol during third-trimester development, a period known as the "brain growth spurt." This period of neurogenesis is characterized by a surge in neuronal proliferation, migration, maturation, and synaptic connectivity. These events occur at different time points across species, however, with the equivalent in rodents occurring within the first two postnatal weeks of life. It has been shown in rodent models of FASD that even a single day of alcohol exposure during the brain growth spurt can induce brain weight reduction and neuronal cell loss, especially in the cerebellum and forebrain structures

(Goodlett et al., 1989, 1990). Yang and colleagues (2014) define this single-binge model as acute alcohol exposure, however, many studies use models of binge-alcohol exposure across several days, achieving high blood-alcohol concentrations (BACs), which can be defined as chronic or subchronic alcohol exposure depending on dosage.

With this latter-type model, previous work from our lab has found persistent effects of alcohol on hippocampal neurogenesis. Alcohol does not seem to inhibit proliferation of new neurons, but does diminish their chances for survival. This decrease in survival rate may be due to a failure to integrate properly into neuronal networks, which is also likely due to decreases found in dendritic complexity. (Klintsova et al., 2007, Hamilton et al., 2010, unpublished data 2015). Alcohol exposure to the developing hippocampus has been shown to induce long-term impairments of spatial learning and memory functions by disruption of neurogenesis, long-term potentiation (LTP), and dendritic development (Berman and Hannigan, 2000; Johnson and Goodlett, 2002; Anderson et al., 2012; Hamilton et al., 2010; Lovinger et al., 1989).

1.2 Role of Microglia in the CNS

Microglia are the resident immune cells of the brain, and, as the primary responders to immune challenges, these phagocytic cells will exhibit various activation states according to the current homeostatic needs of the CNS (Nimmerjahn et al., 2005). Thus, microglia can perform either neurodegenerative actions (classical or M1 macrophage activation) or neuroprotective actions (alternative or M2 activation) with release of associated pro-inflammatory (M1) or anti-inflammatory (M2) cytokines and chemokines. Microglia can exist various morphological states as well: a resting, quiescent state is characterized by a small soma and long thin

processes for surveying adjacent tissue for insults and injury (Fig. 1C). A partial activation state is characterized by an enlarged soma and shortened, thicker processes (Fig. 1D). Finally, a fully-activated or phagocytic state (Fig. 1E) is characterized by a round shape with even shorter processes, or an amoeboid shape (Olah et al., 2011). Upon activation, microglia act as antigen-presenting cells (APCs) and will engulf and phagocytose damaged cells (Fig. 1F) and cellular debris (Aloisi, 2001).

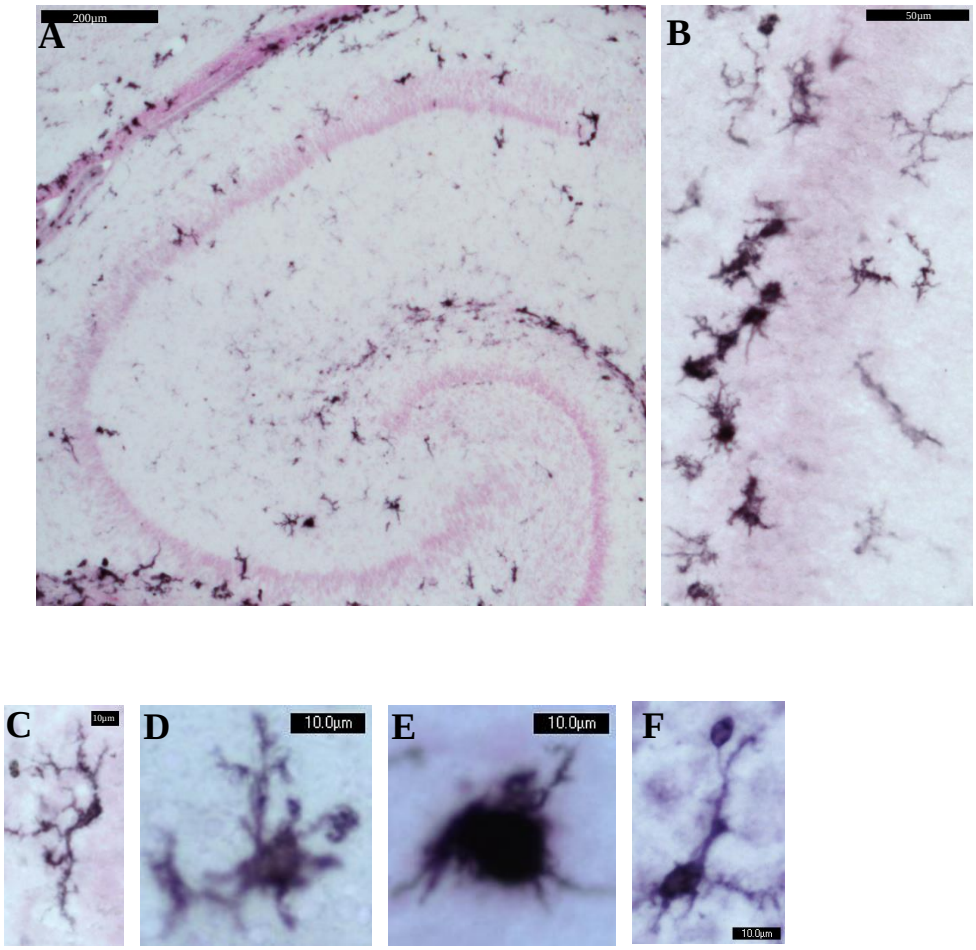

Figure 1: All above representative images taken of microglia within PD5 hippocampus. **A)** 5X zoom of horizontal female hippocampus section. **B)** 20X zoom of male CA1 region, cluster of activated microglia. **C)** 20X zoom of male CA3 region, ramified phenotype. **D)** 40X zoom of male DG, partially-activated phenotype. **E)** 40X zoom of female DG, activated phenotype. **F)** 40X zoom of male CA3 region, microglia with phagosome (active phagocytosis).

As important as microglia are to the immune health of the brain, these cells are not initially generated within the brain itself; the cells originate from macrophage precursors in the peripheral nervous system (PNS) early in development. More specifically, microglia originate from myeloid cells in the bone marrow, which

migrate towards the brain, cross the blood-brain barrier, and finally begin cerebral colonization during embryonic days (ED) 9-10 (Chan, Kohsaka, and Rezaie, 2007; Ginhoux et al., 2010). By ED17, amoeboid-shaped microglia colonize heavily the developing rodent hippocampus, and then by birth, PD0, they begin to develop short, thick processes which then extend even farther by PD4. Finally, by rat adolescence, PD30, microglia begin exhibiting the ramified morphology that persists into and throughout adulthood (Schwarz and Bilbo, 2014) Interestingly, within the developing hippocampus, the dentate gyrus specifically remains devoid of microglial precursors until around the time of birth. (Dalmau et al., 1997).

Microglia serve a diverse array of functions within the brain, especially during neurodevelopmental processes. These phagocytic cells are involved in synaptic pruning during the developmental culling of excess neurons and connections, as well as synapse elimination seen with disease progression (Paolicelli et al., 2011; Stephan et al., 2012). Additionally, microglia play a role in the phagocytosis of newly generated neurons in the hippocampus that are not destined to survive. There is a critical period of cell survival in which neurons can integrate synaptically into neuronal networks, and microglia phagocytose and clear away vulnerable cells before they reach the neuroblast-state (Sierra et al., 2010). Microglia are not inherently "anti-neurogenic," however, and can actually support processes of neurogenesis including proliferation, survival, migration and differentiation. Their functioning is based on the current, homeostatic needs of the brain (Ekdahl et al., 2009). These studies show some of the distinct, essential roles microglia play in the development and maintenance of the CNS. These crucial functions depend on proper activation and regulation of microglia, however, so if aberrant activation occurs, it could lead to persistent and

pronounced detrimental effects. With aberrant activation, microglia can become persistently primed, which can lead to exaggerated immune responses following further insults or the later development of neurodevelopmental or psychopathological disorders, such as schizophrenia and depression (Cai et al., 2000; Meyer et al., 2006; Monji et al., 2009; Miller et al., 2009).

1.3 Alcohol-Induced Activation of Microglia

Alcohol has long been shown to induce structural and synaptic changes, and even death of neurons (Harper, 1998; Paula-Barbosa et al., 1993; Bonthius and West, 1990), but recent studies indicate that microglia are just as susceptible to changes elicited by alcohol exposure. Increased activation of microglia, as well as higher levels of associated cytokines, have been linked with the alcohol-induced neuroinflammatory response in the adult and adolescent brain. At the least, this response is recognized as a consequence of the alcohol exposure, but microglial activation may even be the source of the injury (Marshall et al., 2013). However, the exact order and mechanism still remains unclear. Regardless, neuroinflammation is a necessary result of an immune response following insults and injury of the brain, but prolonged activation of microglia can inflate levels of cytotoxicity by increasing levels of inflammatory cytokines which can then lead to neuronal injury rather than protection and preservation. The response to alcohol is dose-dependent, with a delicate balance between neurodegenerative and protective effects. Following acute or subchronic exposure in adulthood, 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 elicit a partial activation of microglia, characterized by phenotype and an increase in some pro-inflammatory cytokines, but not a full activation (McClain et al., 2011; Marshall et al., 2013). It is likely that higher doses or prolonged exposure to alcohol would induce full, phagocytic activation and increased apoptosis of neurons (McClain et al., 2011). The initial exposure could first serve to prime microglia for further, 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 if a return to homeostasis is not achieved. Potential phenotypic priming of the microglia was demonstrated in the study by Marshall and colleagues (2013), as well by the lasting presence of markers of activation such as cytokine levels, indicating that continued alcohol exposure could lead to an exaggerated immune response resulting in neurodegeneration. Based on these studies, it is likely that developmental alcohol exposure could as well induce activation of microglia that persists into adulthood.

1.4 Rationale for Current Research

Past experiments in the field, like those mentioned above by McClain (2011) and Marshall (2013), have focused mainly on microglial activation following alcohol exposure to the adult and adolescent brain. They demonstrated a shift in microglial morphology to a partially activated state, and persistent elevated levels of pro-inflammatory cytokines. Fewer studies have focused on the effects of neonatal alcohol exposure on microglial activation, however, more have been recently completed. One such study, by Tiwari and Chopra (2011), showed that alcohol exposure on PD7-9 resulted in increased levels of pro-inflammatory cytokines compared to controls when

observed three weeks later on PD28. They did not look at short-term effects but did show that the effects of neonatal alcohol exposure can last at least through adolescence. Other recent studies have begun to focus on the immediate, short-term effects of neonatal exposure. A study on cell cultures from 1-4 day -old mice showed that alcohol reduced viability of cerebellar granule cells as well as microglia, and that this reduction could be prevented with administration of PPAR-y agonists. These agonists may limit alcohol-induced inflammation by suppression of genes that encode pro-inflammatory and cytokines and chemokines (Kane et al., 2011). From our lab, in a recently published work (Boschen et al., 2016), we found that a six-day binge-exposure on PD4-9 resulted in a decrease in microglial cell counts in the CA1 and dentate gyrus of the hippocampus when observed on PD10. However, the rats received alcohol through intragastric intubation across those six days, which may have led to an interacting stress effect. Looking at microglial morphology, alcohol-exposed animals had significantly smaller microglia in all three subfields of the hippocampus, indicating the enhancement of their activation state. Finally, alcohol increased expression of pro-inflammatory cytokine CCL4 and anti-inflammatory TGF-β, while alcohol and intubation both increased pro-inflammatory IL-1β, TNF-α and CD11b. For the latter three cytokines, again, there is a possible stress interaction from the intubation procedure. These data indicate an immediate, inflammatory immune response to alcohol exposure, and possibly an anti-inflammatory coping response to prevent neurotoxicity.

The goal of the current study is to develop a time course of microglial activation in the rat hippocampus following a single-day binge-like alcohol exposure on PD4, and to understand further the neuroimmune response to teratogens in the days

following exposure in both males and females. PD4 was chosen as it is the first day of AE in our six-day binge-model from previous experiments, as well as to compare with current work observing apoptosis following PD4 alcohol exposure. A drive to examine effects on both the male and female brain has been growing in the field of neuroscience and encouraged by the National Institute Health (NIH), and shall be included here. A single-day binge-like alcohol exposure was chosen for this experiment instead of PD4-9 alcohol exposure for two reasons: 1) this model allows us to assess the effects of a single-day of alcohol exposure, which mimics a woman binge-drinking over one weekend during her third-trimester of pregnancy and is very relevant to the human condition, as 1 in 33 pregnant women have reported binge-drinking in a recent study (CDC, 2015), and 2) the single-day exposure potentially minimizes the stress effects of intubation, as the intubations will be limited to one day rather than six days. Considering the potential reduction of stress of the intubation procedure, it is hypothesized that using the single-day model of alcohol exposure will lead to increased proliferation of microglia in the experimental group compared to both control groups, as well as increases in both pro- and anti-inflammatory cytokines, and additionally, a differential effect based on sex will be observed.

Chapter 2

METHODS

2.1 Animals

Seven 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 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. Both male and female pups were used for analyses for this current project, for a combined (immunohistochemistry and gene expression) total of 70 animals. Previously obtained tissue from 11 animals from past experiments was used for preliminary PD8 cell count analysis. 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) on PD4. Specifically, on the single day of AE, rats received 2 alcohol/milk

intubations as well as 2 milk feedings, to account for potential nutrients lost by missed suckling from the dam. 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 ensure proper healthy development. On PD5 or PD8, 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, when peak BACs are typically found, 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 Immunocytochemistry

Animals were anesthetized with injections of ketamine/xylazine cocktail and subsequently trancardially 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

horizontally at 40 µm on a cryostat at -20°C, throughout the entire hippocampus and stored at 20°C in cryoprotectant solution (Ethylene glycol and sucrose in 0.2M PBS). A systematic random sampling procedure, every 12th section, was used in selecting the sections for processing.

Microglial cells were identified by immunocytochemical staining with microglial-specific marker Ionized calcium-Binding Adaptor molecule 1 (Iba-1) antibodies. Sections were washed three times in 50mM TRIS-buffer solution (TBS) and then incubated in 2% H2O2 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), hydrogen peroxide and nickel-enhanced diaminobenzidine (DAB).

Additionally, a sampling of sections was labeled with the inflammatory marker OX-6 to observe inflammatory Major Histocompatibility Complex-II (MHC-II) using a protocol obtained from Dr. Gary Wenk of Ohio State University, and adapted for our uses. Sections were washed three times in .1M phosphate-buffer solution (PBS) and

then incubated in 0.3% H2O2 in 50% methanol for one hour to eliminate endogenous peroxidases. Sections were again rinsed three times in .1M PBS. Non-specific binding sites were blocked by incubation for 1 hour in 5% normal goat serum (Sigma Life Sciences) in PBS. Then sections were transferred into primary antibody anti-OX-6 made in mouse (1:200; BD Bioscience) in blocking solution, and incubated for 72 hours at 4°C. Sections were then washed three times in .1M PBS to rinse off the primary antibody before entering incubation in secondary antibody (anti-mouse IgG made in goat, 1:200, Vector Laboratories Inc.) for 2 hours at room temperature in washing solution (5% goat serum in PBS). The tissue was then rinsed again 3 times in PBS before being transferred to ABC solution (Vector Laboratories, Burlingame, CA) for 1 hour to amplify the signal. Finally, localization of binding sites was 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). Each region was divided into three layers of tracings: for DG: granule cell layer, hilus, and molecular layer; for CA1/CA3 regions: pyramidal cell layer: stratum oriens, and stratum radiatum. 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 of interest on each section. For cell number quantification, the following parameters were used: sampling grid frame of 200 X 200

micrometers, counting frame of 200 X 200 micrometers, a guard zone of 2 micrometers, and a disector 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 was computed with the formula in Figure 2. The mean coefficient of error (CE) for the number of cells did not exceed the recommended m = 0.1.

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

Figure 2: Stereological Estimation Formula. (Q = # of counted cell markers, t = section thickness, h = dissector height, asf = area sampling fraction; ssf = section sampling fraction).

2.6 Polymerase Chain Reaction (PCR)

Following rapid decapitation on PD5, brains were cooled with 2-methylbutane on dry ice, and 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 40 animals using real-time PCR (Bio-Rad CFX96) with Taqman probes (Thermofisher Scientific) and forward and reverse primers for the IL-1β gene and the CCL4 gene. Tubulin was used as a reference gene. All reactions for each gene target and reference were run in

triplicate. Product specificity was assessed gel electrophoresis. 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

For postnatal days 4 and 5, one-way ANOVAs were performed to compare weights of animals within neonatal conditions on those days. For microglial cell counts, two-way ANOVAs were performed with the factors of sex by neonatal treatment. When appropriate, Fisher's LSD post hoc tests were run. Two-tailed T-tests were used to compare sex differences within regions. 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 (BACs)

All animals were weighed on postnatal days 4 and 5 (before intubation and sacrifice, respectively). Table 1 shows the average weights for each condition at each age the animals were weighed. No significant effect of condition on average weight was found on either day (p = NS).

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	10.94 ± 0.20	10.72 ± 0.18	10.83 ± 0.14
PD5	12.67 ± 0.29	12.41 ± 0.17	12.21 ± 0.18
BAC			----

3.2 PD5 Iba-1+ Cell Counts

Microglial cell counts were determined using unbiased stereological analysis of Iba-1+ cells in the hippocampus of rats sacrificed on PD5. Figure 1 depicts representative images of the PD5 hippocampus and various microglial activation states and actions. In the dentate gyrus (Fig. 3), 2-way ANOVAs revealed a main interaction of sex and condition in the molecular layer ($F(2,30)=4.325$, $p= 0.024$) as well as in the

hilus ($F(2,30)=3.859$, $p= 0.035$). In addition, main effects of sex in the molecular ($F(1,30)=15.632$, $p= 0.001$) and granule cell ($F(1,30)=5.139$, $p= 0.032$) layers were found. For males, in the molecular layer (Fig. 3C) and the hilus (Fig.3A) of the DG, Fisher's LSD revealed that rats in the AE group had significantly higher cell counts when compared with SC ($p = 0.036$ and $p = 0.039$, respectively). For females, in the molecular layer (Fig. 3C) and the granule cell layer (Fig. 3B) of the DG, Fisher's LSD revealed that rats in the AE group had significantly higher cell counts when compared with rats in the SI group ($p = 0.039$ and $p = 0.013$, respectively). Two-tailed *t*-tests found significant sex differences in the following regions and conditions: molecular layer (Fig. 3C; AE rats, $p = 0.001$, SI rats, $p = 0.05$); granule cell layer (Fig. 3B; AE rats, $p = 0.023$); and hilus (Fig. 3A; AE rats, $p = 0.01$).

In CA3 (Fig. 4), two-way ANOVAs revealed main effects of sex in the stratum oriens ($F(1,30)=9.001$, $p= 0.006$), pyramidal cell layer ($F(1,30)=8.689$, $p= 0.007$), and stratum radiatum ($F(1,30)=6.550$, $p= 0.017$). No main effect of condition, or interaction of sex and condition was found. Two-tailed *t*-tests found significant sex differences in the following regions and conditions: stratum oriens (Fig. 4A; AE rats, $p = 0.023$) and pyramidal cell layer (Fig. 4B; SI rats, $p = 0.033$).

In CA1 (Fig. 5), two-way ANOVAs revealed a main effect of sex in the stratum oriens ($F(1,30)=7.919$, $p= 0.009$), pyramidal cell layer ($F(1,30)=61.872$, $p < 0.001$), and stratum radiatum ($F(1,30)=8.676$, $p= 0.007$). No main effect of condition, or interaction of sex and condition was found. Two-tailed T-tests found significant sex differences in the pyramidal cell layer (Fig. 5B): AE rats, $p = 0.030$; SI rats, $p = 0.040$).

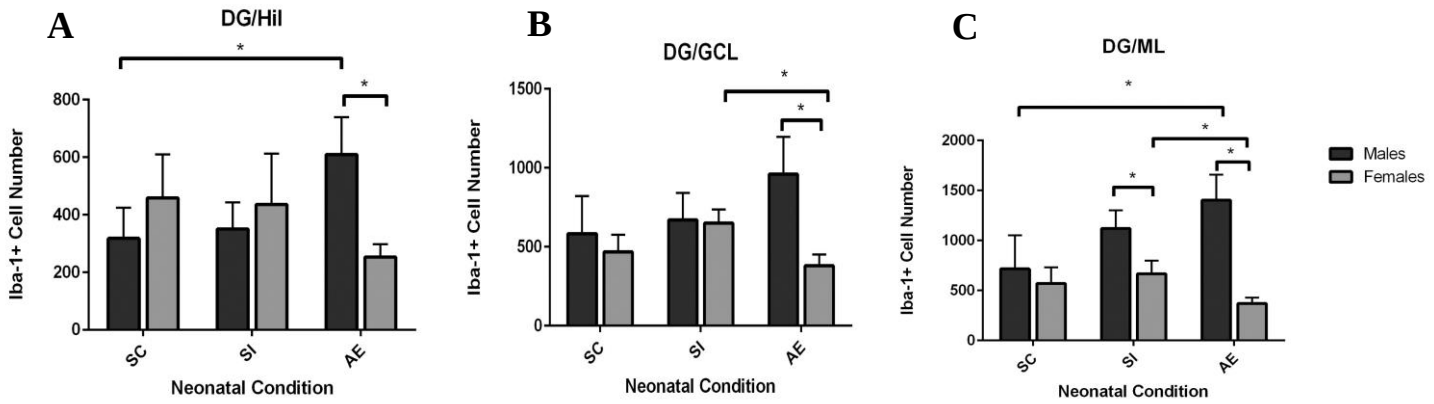

Figure 3: PD5 Iba-1+ cell counts in Dentate Gyrus. **A)** Hilus, **B)** granule cell layer, and **C)** molecular layer. ** = $p \leq .001$ * = $p < 0.05$, SC = suckle control, SI = sham intubated, AE = alcohol exposed. All data are expressed as mean ± standard error of the mean (SEM).

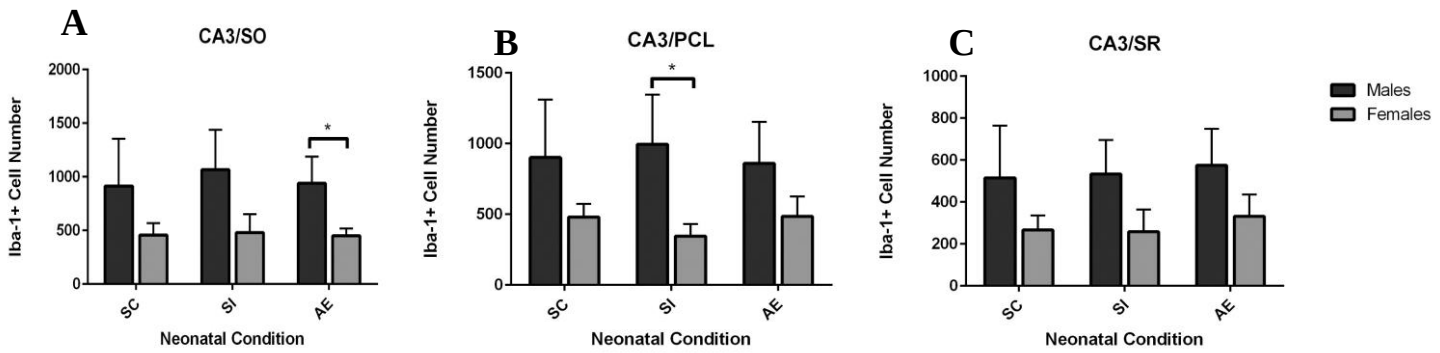

Figure 4: PD5 Iba-1+ cell counts in CA3 **A)** stratum oriens, **B)** pyramidal cell layer, **C)** stratum radiatum. * = $p < .05$, SC = suckle control, SI = sham intubated, AE = alcohol exposed. All data are expressed as mean ± standard error of the mean (SEM)

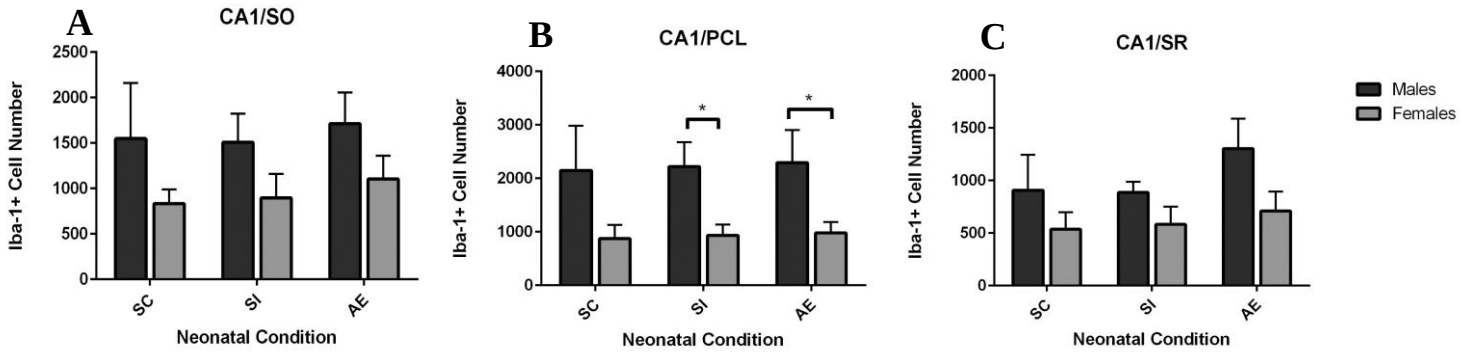

Figure 5: PD5 Iba-1+ cell counts in CA1 **A)** stratum oriens, **B)** pyramidal cell layer, **C)** stratum radiatum. * = $p < .05$, SC = suckle control, SI = sham intubated, AE = alcohol exposed. All data are expressed as mean ± standard error of the mean (SEM)

3.3 PD8 Iba-1+ Microglia Cell Counts

Microglial cell counts were determined using unbiased stereological analysis of Iba-1+ cells in the hippocampus on PD8, as seen in Figures 6-8. The following data is preliminary and consists of 11 animals: In the dentate gyrus (Fig. 6), two-way ANOVAs revealed a main effect of sex in the granule cell layer (p = .001). In CA3 (Fig. 7), 2-way ANOVAs revealed main effects of sex in stratum oriens (Fig. 7A; p = .0001), pyramidal cell layer (Fig. 7B; p=.022), and stratum radiatum (Fig. 7C; p = .006), and a main effect of condition in stratum oriens (Fig. 7A; p = .032). In CA1 (Fig. 8), two-way ANOVAS revealed a main effect of sex in stratum oriens (Fig. 8A; p = .001), and a main effect of sex*condition in the pyramidal cell layer (p = .026).

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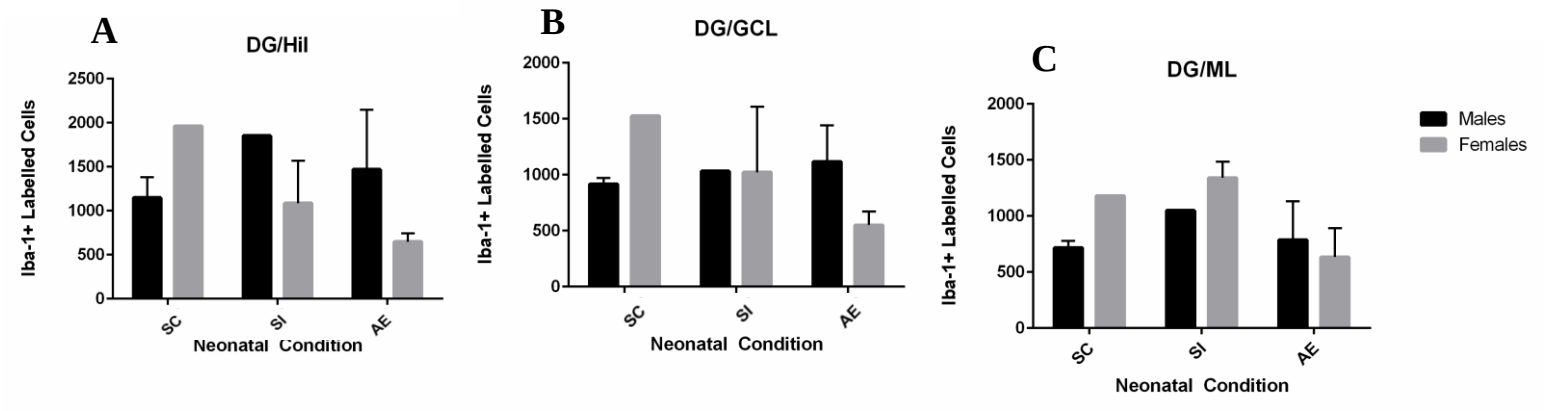

Figure 6: PD8 Iba-1+ cell counts in Dentate Gyrus **A)** Hilus, **B)** granule cell layer, **C)** molecular layer. SC = suckle control, SI = sham intubated, AE = alcohol exposed. All data are expressed as mean ± standard error of the mean (SEM)

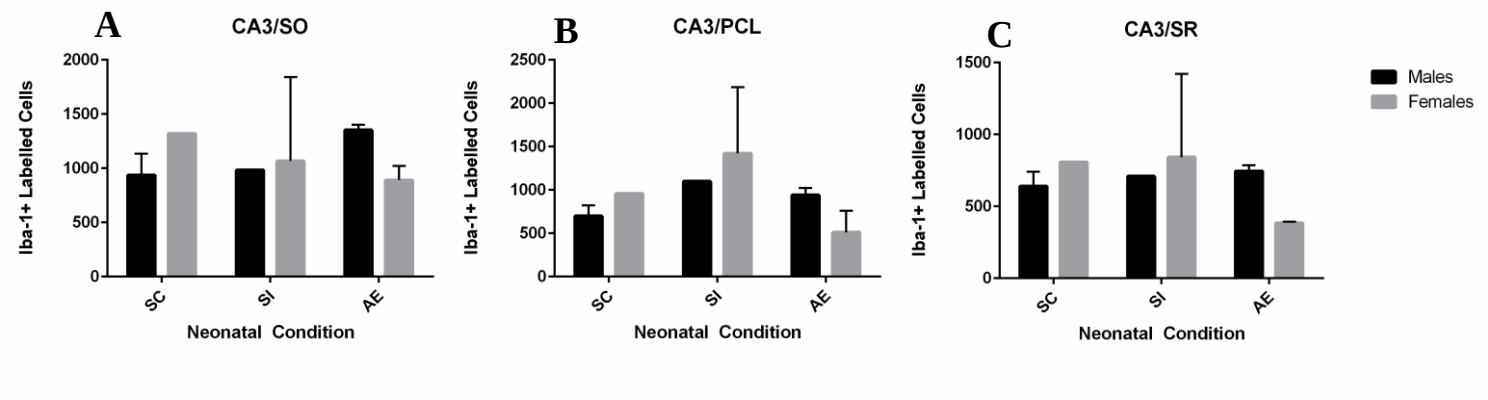

Figure 7: PD8 Iba-1+ cell counts in CA3 **A)** stratum oriens, **B)** pyramidal cell layer, **C)** stratum radiatum SC = suckle control, SI = sham intubated, AE = alcohol exposed. All data are expressed as mean ± standard error of the mean (SEM)

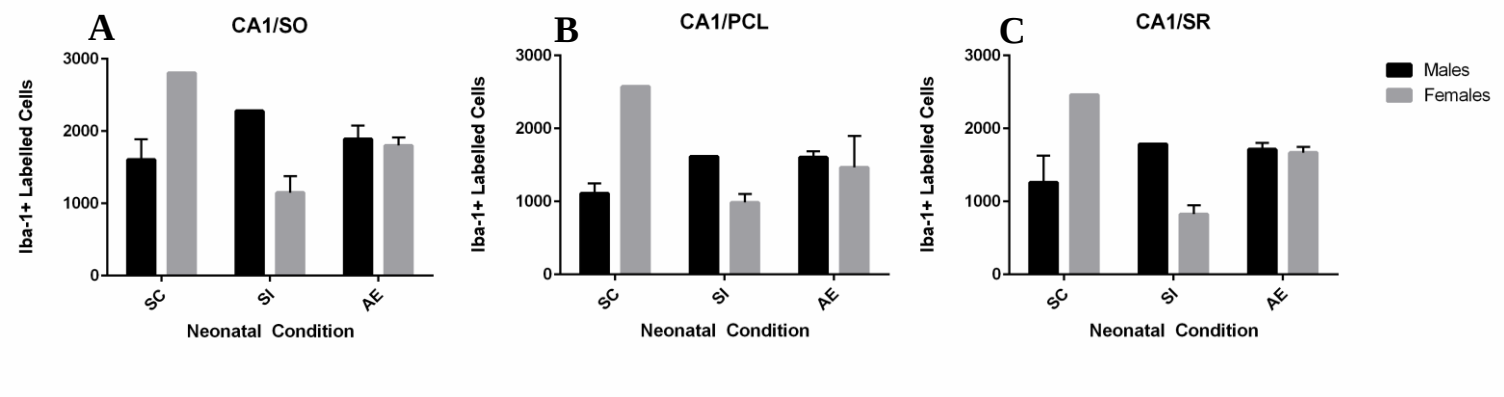

Figure 8: PD8 Iba-1+ cell counts in CA1 **A)** stratum oriens, **B)** pyramidal cell layer, **C)** stratum radiatum. SC = suckle control, SI = sham intubated, AE = alcohol exposed. All data are expressed as mean ± standard error of the mean (SEM)

3.4 MHC-II Immunohistochemistry

MHC-II expression was not observed through OX-6 labelling in either PD5 or PD8 brain sections in any of the experimental conditions. Adult (3 months and 6 months) brain tissue treated with known immune-activator lipopolysaccharide (LPS) was obtained from Dr. Gary Wenk's lab at Ohio State University, and labelled using the same OX-6 procedure. MHC-II expression was widely observed within this tissue. Previous research has shown the appearance of OX-6 (MHC-II) expression to be around embryonic day (ED)14 and increase through ED20, and that postnatal expression is hardly detectable unless stimulated by LPS or IFN-γ (Wang et al., 1996; Xu & Ling 1994). This may further explain the absence of MHC-II positive cells we observed.

3.5 Cytokine Gene Expression

Real-time quantitative Polymerase chain reaction (PCR) was performed to assess the levels of mRNA gene expression of the associated pro-inflammatory cytokines CCL4 and IL-1β, as seen in Figure 9. Two-way ANOVAs did not reveal statistically significant effects of sex or condition for either CCL4 or IL-1β, but a trending main effect of condition was seen for CCL4 (Fig. 9A; $p = .075$).) Additional animals are currently being generated for immediate future analysis to supplement this data into expected significance.

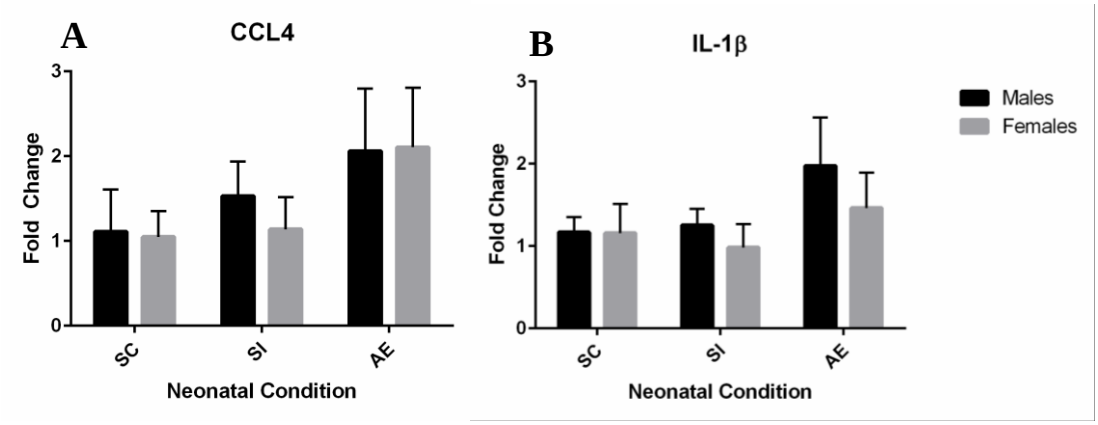

Figure 9. PD5 Cytokine Gene Expression **A)** CCL4 **B)** IL-1ß. SC = suckle control, SI = sham intubated, AE = alcohol exposed. All data are expressed as mean ± standard error of the mean (SEM)

Chapter 4

DISCUSSION

4.1 Summary of Results

This study set out to add data to a timeline of microglial activation in the developing rodent brain, by observing the effects of a single-day binge-alcohol exposure on microglial response during the third-trimester equivalent. We found that 24 hours after the exposure alcohol had differential effects on PD5 microglial cell counts in male vs female dentate gyrus, CA1, and CA3 subregions of the developing hippocampus, supporting our hypothesis. In the molecular layer and hilus of the DG, alcohol was found to increase cell counts in males, compared to SC; in the molecular layer and granule cell layer of the DG, alcohol was found to decrease cell counts in females, compared to SI, showing an overall opposite response. A main effect of sex, with males having higher microglial cell counts compared to females, was found in all hippocampal subregions, except the hilus of the DG. PD8 data is very preliminary, but similar trends are seen thus far. Additionally, the increases in the pro-inflammatory cytokines CCL4 and IL-1β in alcohol exposed animals were not statistically significant compared to controls, but trending increases were seen in the AE group compared to SC for CCL4 when sexes were combined. Additional animals must be added for gene expression data to reach full significance.

4.2 Iba-1+ Cell Counts

Significant sex-dependent differences in cell number were found in all hippocampal regions (main effects of sex), except the hilus of the dentate gyrus. In the regions excluding the hilus, males had higher levels of microglia than females. It is

likely that, at this point in development, microglia are colonizing specific brain regions at different rates before levels even out later in development. In subregions of the dentate gyrus, sex-specific effects based on condition were observed. In both the molecular layer and the hilus, males in the AE group had significantly higher levels of microglia compared to suckle controls, but females in the AE group had the opposite effect: significantly lower levels of cell counts compared to sham-intubated controls. Males and females are clearly reacting to alcohol exposure in different ways, but the reason why remains to be elucidated. One possibility for this sex-specific alcohol effect differences may be due to which brain areas are currently colonized by microglia at this time point and which specific brain regions may be preferentially targeted by alcohol. Males already seem to have higher levels of microglia in the hippocampus at this time point, and if challenged by an immune response, more microglia may be being recruited from other adjacent brain regions to the hippocampus. Females have lower levels of microglia in the hippocampus at this time point in development, and even fewer after alcohol exposure, so it is possible that the cells present in the hippocampus were recruited to another brain region that was being more adversely affected.

4.3 Cytokine Gene Expression

While no significant results were obtained thus far, trending increases were seen in the alcohol exposed groups regardless of sex for both of the pro-inflammatory markers CCL4 and IL-1ß. With the addition of more animals per group, significant results are expected as we have reported AE-specific increases in CCL4 expression, and increases in IL-1ß in AE and SI animals with our work with PD10 animals (Boschen et al., 2016). It is predicted that with the reduced stress of the single-day

binge model, the increase in IL-1ß expression will be AE-specific. Chemokines like CCL4 mediate inflammation, and are involved in activating the specific immune response, and by doing so can upregulate expression of other inflammatory cytokines during an immune response such as IL-1ß (Quandt & Dorovini-Zis, 2004; Machado et al., 2015). IL-1ß is also a well-known mediator of inflammation. IL-1ß activation is tightly controlled; the inactive precursor must be cleaved by the protease caspase-1. Chronic and aberrant activation of IL-1ß has been implicated in many diseases, such as Alzheimer's disease, diabetes, multiple sclerosis and stroke, and stroke (Dinarello 2011; Shaftel et al., 2008; Isik et al., 2013)

4.4 Relevance to Previous Work

Recently published work from our lab had demonstrated that a six-day binge-exposure from PD4-9 led to decreases in microglial cell counts in the CA1 and dentate gyrus of the male hippocampus when observed on PD10 (Boschen et al., 2016). This decrease was unexpected, as studies in the adult and adolescent brain had showed increases in microglial cell counts in alcohol-exposed animals (Marshall et al., 2013; McClain et al., 2011). However, we noted this decrease may have been due to the stress from six consecutive days of intubation, as our lab as shown increased levels of plasma corticosterone in both SI and AE animals (Boschen et al., 2015). We limited our current study to only one day of intubation to reduce the potential stress effect from our model, and an increase in cell counts has now been seen in the male brain, suggesting a similar effect that has been shown in adulthood. Upon analyzing corticosterone levels, we can more definitively confirm this. Drew and colleagues (2015) used a very similar FASD model with a PD4-9 alcohol exposure, but in mice, with similar results to ours and without any conflicting effects of stress. Following

alcohol exposure, clear increases of IL-1β and TNF-α were observed in the hippocampus, cerebellum and cerebral cortex, and increased CCL2 expression in the hippocampus and cerebellum. No stress effects of their intra-esophageal gavage were noted. Microglial activation was also increased, classified by decreases in cell territory, as retraction of processes indicates an activated state. Another model of FASDs, using a vapor chamber, found that AE from PD3-5 caused neurodegeneration of cerebellar purkinje cells when assessed on PD6 and PD45, but not hippocampal neurons. Increased levels of pro-inflammatory cytokines were seen during alcohol withdrawal in both the hippocampus and cerebellum. These results suggest time and brain region dependent microglial response and effect (Topper et al., 2015)

Our lab also focuses on the long-term survival of newly generated neurons following a similar third-trimester equivalent binge-alcohol model, and based on new findings, it is likely that microglia play some role in what we have previously found in our research. We have found that alcohol-exposure negatively impacts the survival of new neurons born in the dentate gyrus of the hippocampus, and may also inhibit their synaptic integration into neuronal networks through decreases in dendritic material (Klintsova et al., 2007; unpublished data 2015). Interventions employed to ameliorate the effects of alcohol include wheel-running (WR) exercise and environmental complexity (EC), both of which have specific effects on reducing hippocampal-dependent impairments that are associated with FASDs. WR has been shown to increase neuronal proliferation in both control and AE rats, while EC following WR exercise enhanced the long-term survival of adult-born cells (Hamilton et al., 2012). Based on results from our work on the alcohol-induced immune response, it would be possible that activation of microglia may lead to long-term alterations to the

neuroimmune system that contribute to reductions the survival and integration of new neurons, as well as be involved with positive effects of WR and EC through an anti-inflammatory, homeostatic response. This neuroprotective response may be the result of microglial-derived BDNF as activation has been shown to cause its release (Gomes et al., 2013). Previous work in our lab has shown increases in both protein levels and mRNA expression of BDNF in the hippocampus following alcohol exposure, thus supporting this as a possibility (Boschen et al., 2015).

Interesting work from other labs support these ideas as well. The production of pro-inflammatory mediators has been shown to disrupt the delicate balance that is needed for proper neuronal functioning and communication, leading to detrimental effects on neuroplasticity and neurogenesis (Yirmiya & Goshen 2010). These changes to the neuroimmune system can be long-lasting, exhibited by a disrupted balance in the immune response. Levels of pro-inflammatory cytokines remain elevated following alcohol exposure, while consistent decreases are seen in anti-inflammatory cytokine production (Cui et al., 2014; Qin et al., 2008). However, upon withdrawal and abstinence from alcohol, an increase proliferation of neurotrphin-releasing microglia is seen, as well as reductions in ethanol-induced cognitive dysfunction, indicating a regenerative role of microglia, and drive for homeostatic reestablishment, after chronic alcohol exposure (Chastain & Sarkar 2014; Zhao et al., 2013) A study by Nixon and colleagues (2008) correlated a responsive proliferation of microglia with a surge in neuronal proliferation two days following alcohol exposure, furthering supporting a neurotrophic response of microglia on the path of neuronal recovery.

4.5 Relevance to FASD in Humans

Continuing to perform studies with rodent models will further our understanding of the effects of alcohol on the developing brain and the neuroimmune response. While the human and rodent CNS may not be completely analogous, it is reasonable to expect that the major innate immune responses are similar. Studies of alcoholism in humans have shown an increased toxicity to CCL4 and enhanced conversion to its toxic free radical, likely due to a decline in the activity of alcohol dehydrogenase (Zimmerman 1978), and also shown higher concentrations of inflammatory cytokines such as IL-1β in the blood-plasma (Achur et al., 2010). Correlations of serum levels of cytokines and alcohol craving have also been shown, but how altered cytokine levels contribute directly, if at all, to dependence remains to be seen (Leclercq et al., 2014). Additionally, a study by Schultz and colleagues (2004) showed that infants have a decreased capacity to elicit an anti-inflammatory response. Blood samples treated with LPS showed lower production of anti-inflammatory IL-10 and less inhibition of pro-inflammatory TNF-α when compared to adults, which indicates a weak compensatory response that could fail to initiate a crucial return to homeostasis. Uncovering the effects of alcohol on the microglial-mediated immune response, both neurodegenerative and neuroprotective, may lead to future treatment of those suffering from FASDs. A corrected homeostatic drive may reduce deficits due to neurodegeneration in these patients.

4.6 Conclusions and Future Directions

This study sought to further elucidate the effects of developmental alcohol exposure on microglial activation. It was predicted that using a single-day binge model would reduce potential stress effects, and implicate a stronger alcohol effect. Data so

far suggest that this was successful: In the PD5 male hippocampus, cell counts were increased following alcohol exposure, instead of decreased as seen before in our PD10 animals following six-day binge exposure, and trending increases were seen in CCL4 and IL-1ß expression in the AE group. Sex differences revealed overall higher levels of microglial cell counts in the male hippocampus as compared to females, and an additional opposite effect in the female dentate gyrus: a decrease in cell counts following alcohol exposure. To strengthen these results and draw more significant conclusions, more animals must and will be added.

Some future directions will be carried out shortly, which include looking at PD8 cytokine gene expression, as well as the addition of anti-inflammatory cytokine analysis. It is imperative to see how the balance of pro- and anti-inflammatory markers may be disrupted following alcohol, and if this occurs in a sex-dependent manner. One way to further this line of research would be to observe the long-term effects of developmental exposure. The priming of microglia during the first insult in development may lead to long-term, aberrant activation and a disruption of homeostatic drive when further insults are introduced in adulthood. Additionally, the potential role of microglia in apoptosis should be investigated through analysis of IGF-1 expression. Microglial derived IGF-1 promotes cell differentiation and survival (O'Kusky et al., 2000; Butovsky et al., 2006, Ueno et al., 20013). Alcohol exposure has been shown to reduce IGF-1 receptor functioning and serum levels, (Resnicoff et l.,1993; Rojdmark et al., 2000; Lavigne et al., 2005), and wheel running in mice has been shown to increase IGF-1 expression (Kohman et al. 2012). I believe that based on some of these previous studies, microglia may be directly involved with the cell death, survival, and rescue in our alcohol models.

In summary, the current study suggests microglial involvement in alcohol-induced hippocampal neurodegeneration, and that this involvement is sex-specific. While much remains to be discovered, valuable information has been added to the understanding of FASDs and the developmental neuroimmune response.

REFERENCES

Achur, R., Freeman, W., & Vrana, K. (2010). Circulating Cytokines as Biomarkers of Alcohol Abuse and Alcoholism. Journal of NeuroImmune Pharmacology, 5(1), 83-91.

Aloisi, F. (2001). Immune function of microglia. *Glia, 36*(2), 165-179.

Anderson, M., Nokia, M., Govindaraju, K., & Shors, T. (2012). Moderate drinking? Alcohol consumption significantly decreases neurogenesis in the adult hippocampus.*Neuroscience,* 202-209.

Bailey, B. A., & Sokol, R. J. (2008). Pregnancy and Alcohol Use: Evidence and Recommendations for Prenatal Care. *Clinical Obstetrics and Gynecology, 51*(2), 436-444. doi:10.1097/grf.0b013e31816fea3d

Berman R, Hannigan J. (2000) Effects of prenatal alcohol exposure on the hippocampus: spatial behavior, electrophysiology and neuroanatomy. *Hippocampus* 10:94–110.

Bonthius, D., & West, J. (1990). Alcohol-Induced Neuronal Loss in Developing Rats: Increased Brain Damage with Binge Exposure. *Alcoholism: Clinical and Experimental Research Alcoholism Clin Exp Res, 14*(1), 107-118.

Boschen, K., Criss, K., Palamarchouk, V., Roth, T., & Klintsova, A. (2015). Effects of developmental alcohol exposure vs. intubation stress on BDNF and TrkB expression in the hippocampus and frontal cortex of neonatal rats. International Journal of Developmental Neuroscience, 43, 16-24.

Boschen, K. E., Ruggiero, M. J., & Klintsova, A. Y. (2016). Neonatal binge alcohol exposure increases microglial activation in the developing rat hippocampus. *Neuroscience*.

Butovsky, O., Ziv, Y., Schwartz, A., Landa, G., Talpalar, A. E., Pluchino, S., . . . Schwartz, M. (2006). Microglia activated by IL-4 or IFN-γ differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. *Molecular and Cellular Neuroscience, 31*(1), 149-160. doi:10.1016/j.mcn.2005.10.006

Cai, Z., Pan, Z., Pang, Y., Evans, O., & Rhodes, P. (2000). Cytokine Induction in Fetal Rat Brains and Brain Injury in Neonatal Rats after Maternal Lipopolysaccharide Administration. *Pediatric Research, 47*(1), 64-64.

Chan, W., Kohsaka, S., & Rezaie, P. (2007). The origin and cell lineage of microglia—New concepts. *Brain Research Reviews, 53*(2), 344-354.

Chastain, L. G., & Sarkar, D. K. (2014). Role of Microglia in Regulation of Ethanol Neurotoxic Action. *International Review of Neurobiology Neuroimmune Signaling in Drug Actions and Addictions,* 81-103. doi:10.1016/b978-0-12-801284-0.00004-x

CDC. (2015). Fetal Alcohol Syndrome Among Children Aged 7–9 Years — Arizona, Colorado, and New York, 2010. *Morbidity and Mortality Weekly Report (MMWR), 64*(03), 54-57.

CDC. (2015). Alcohol Use and Binge Drinking Among Women of Childbearing Age – United States, 2011-2013. *Morbidity and Mortality Weekly Report (MMWR), 64*(37), 1042-1046.

Cui, C., Shurtleff, D., & Harris, R. A. (2014). Neuroimmune Mechanisms of Alcohol and Drug Addiction. *International Review of Neurobiology Neuroimmune Signaling in Drug Actions and Addictions,* 1-12. doi:10.1016/b978-0-12-801284-0.00001-4

Dalmau, I., Finsen, B., Tander, N., Zimmer, J., Gonzalez, B., & Castellano, B. (1997). Development of microglia in the prenatal rat hippocampus. *The Journal of Comparative Neurology J. Comp. Neurol., 377*(1), 70-84. doi:10.1002/(sici)1096-9861(19970106)377:13.0.co;2-g

Dinarello, C. A. (2011). A clinical perspective of IL-1β as the gatekeeper of inflammation. *European Journal of Immunology Eur. J. Immunol., 41*(5), 1203-1217.

Drew, P. D., Johnson, J. W., Douglas, J. C., Phelan, K. D., & Kane, C. J. (2015). Pioglitazone Blocks Ethanol Induction of Microglial Activation and Immune Responses in the Hippocampus, Cerebellum, and Cerebral Cortex in a Mouse Model of Fetal Alcohol Spectrum Disorders. *Alcohol Clin Exp Res Alcoholism: Clinical and Experimental Research, 39*(3), 445-454. doi:10.1111/acer.12639

Ekdahl, C., Kokaia, Z., & Lindvall, O. (2009). Brain inflammation and adult neurogenesis: The dual role of microglia. *Neuroscience, 158*(3), 1021-1029.

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., . . . Merad, M. (2010). Fate Mapping Analysis Reveals That Adult Microglia Derive from Primitive Macrophages. *Science,*841-845.

Gomes, C., Ferreira, R., George, J., Sanches, R., Rodrigues, D., Gonçalves, N., &

Cunha, R. (2013). Activation of microglial cells triggers a release of brain-derived neurotrophic factor (BDNF) inducing their proliferation in an adenosine A2A receptor-dependent manner: A2A receptor blockade prevents BDNF release and proliferation of microglia. Journal of Neuroinflammation, 10, 16-16.

Goodlett, C., Mahoney, J., & West, J. (1989). Brain growth deficits following a single day of alcohol exposure in the neonatal rat. Alcohol, 6(2), 121-126.

Goodlett, C., Marcussen, B., & West, J. (1990). A single day of alcohol exposure during the brain growth spurt induces brain weight restriction and cerebellar Purkinje cell loss. Alcohol, 7(2), 107-114.

Green, C., Mihic, A., Nikkel, S., Stade, B., Rasmussen, C., Munoz, D., & Reynolds, J. (2009). Executive function deficits in children with fetal alcohol spectrum disorders (FASD) measured using the Cambridge Neuropsychological Tests Automated Battery (CANTAB). Journal of Child Psychology and Psychiatry, 50(6), 688-697.

Hamilton, G., Whitcher, L., & Klintsova, A. (2010). Postnatal binge-like alcohol exposure decreases dendritic complexity while increasing the density of mature spines in mPFC Layer II/III pyramidal neurons. *Synapse, 64*(2), 127-135.

Hamilton, G., Boschen, K., Goodlett, C., Greenough, W., & Klintsova, A. (2012). Housing in Environmental Complexity Following Wheel Running Augments Survival of Newly Generated Hippocampal Neurons in a Rat Model of Binge Alcohol

Harper, C. (1998). The Neuropathology of Alcohol-specific Brain Damage, or Does Alcohol Damage the Brain? *Journal of Neuropathology and Experimental Neurology, 57*(2), 101-110.

Isik, N., Arman, A., Canturk, I. A., Gurkan, A. C., Candan, F., Aktan, S., . . . List, E. O. (2013). Multiple sclerosis: Association with the interleukin-1 gene family polymorphisms in the Turkish population. *International Journal of Neuroscience, 123*(10), 711-718. doi:10.3109/00207454.2013.795563

Johnson T, Goodlett C (2002) Selective and enduring deficits in spatial learning after limited neonatal binge alcohol exposure in male rats. *Alcohol Clin Exp Res* 26:83–89.

Kane, C. J., Phelan, K. D., Han, L., Smith, R. R., Xie, J., Douglas, J. C., & Drew, P. D. (2011). Protection of neurons and microglia against ethanol in a mouse model of fetal alcohol spectrum disorders by peroxisome proliferator-activated receptor-γ agonists. *Brain, Behavior, and Immunity, 25*. doi:10.1016/j.bbi.2011.02.016

Klintsova, A.Y., Helfer, J.L., Calizo, L.H., Dong, W.K., Goodlett, C.R., Greenough, W.T., 2007. Persistent impairment of hippocampal neurogenesis in young adult rats following early postnatal alcohol exposure. Alcohol Clin. Exp. Res. 31, 2073–2082.

Kohman, R. A., Deyoung, E. K., Bhattacharya, T. K., Peterson, L. N., & Rhodes, J. S. (2012). Wheel running attenuates microglia proliferation and increases expression of a proneurogenic phenotype in the hippocampus of aged mice. *Brain, Behavior, and Immunity, 26*(5), 803-810. doi:10.1016/j.bbi.2011.10.006

Lavigne, J. A., Baer, D. J., Wimbrow, H. H.…Taylor, P. R. (2005). Effects of alcohol on insulin-like growth factor I and insulin-like growth factor binding protein 3 in postmenopausal women. *American Society for Clinical Nutrition*.

Leclercq, S., Saeger, C. D., Delzenne, N., Timary, P. D., & Stärkel, P. (2014). Role of Inflammatory Pathways, Blood Mononuclear Cells, and Gut-Derived Bacterial Products in Alcohol Dependence. *Biological Psychiatry, 76*(9), 725-733. doi:10.1016/j.biopsych.2014.02.003

Lebel, C., Rasmussen, C., Wyper, K., Walker, L., Andrew, G., Yager, J., & Beaulieu, C. (2008). Brain Diffusion Abnormalities in Children With Fetal Alcohol Spectrum Disorder. Alcoholism: Clinical and Experimental Research, 32(10), 1732-1740.

Lovinger, D., White, G., & Weight, F. (1989). Ethanol Inhibits NMDA-activated Ion Current In Hippocampal Neurons. *Science,* 1721-1724.

Lupton, C., Burd, L., & Harwood, R. (2004). Cost Of Fetal Alcohol Spectrum Disorders. *American Journal of Medical Genetics, 127*(1), 42-50.

Machado, F. D., Marinho, J. P., Abujamra, A. L., Dani, C., Quincozes-Santos, A., & Funchal, C. (2015). Carbon Tetrachloride Increases the Pro-inflammatory Cytokines Levels in Different Brain Areas of Wistar Rats: The Protective Effect of Acai Frozen Pulp. *Neurochem Res Neurochemical Research, 40*(9), 1976-1983.

Marshall, S., Mcclain, J., Kelso, M., Hopkins, D., Pauly, J., & Nixon, K. (2013). Microglial activation is not equivalent to neuroinflammation in alcohol-induced neurodegeneration: The importance of microglia phenotype. *Neurobiology of Disease, 54*, 239-251.

Mattson SN, Riley EP. A review of the neurobehavioral deficits in children with fetal alcohol syndrome or prenatal exposure to alcohol. Alcoholism: Clinical and Experimental Research. 1998;22(2):279–294.

Mcclain, J., Morris, S., Deeny, M., Marshall, S., Hayes, D., Kiser, Z., & Nixon, K. (2011). Adolescent binge alcohol exposure induces long-lasting partial activation of microglia.*Brain, Behavior, and Immunity*, S120-S128.

McKeown, S.M. (2015). Influence of Wheel Running and Environmental Complexity on Adult Hippocampal Neurogenesis in Rats Postnatally Exposed to Alcohol. *Unpublished manuscript*.

Meyer, U., Feldon, J., Schedlowski, M., & Yee, B. (2006). Immunological Stress At The Maternal–foetal Interface: A Link Between Neurodevelopment And Adult Psychopathology. *Brain, Behavior, and Immunity, 20*(4), 378-388.

Miller, A., Maletic, V., & Raison, C. (2009). Inflammation and Its Discontents: The Role of Cytokines in the Pathophysiology of Major Depression. *Biological Psychiatry, 65*(9), 732-741

Monji, A., Kato, T., & Kanba, S. (2009). Cytokines and schizophrenia: Microglia hypothesis of schizophrenia. *Psychiatry and Clinical Neurosciences, 63*(3), 257-265.

NIH Fact Sheets - Fetal Alcohol Spectrum Disorders. (2013, March 29). Retrieved July 22, 2015.

National Institute of Mental Health (2011) *Workshop*, Sex differences in brain, behavior, mental health and mental disorders, (Bethesda, MD) http://www.nimh.nih.gov/research-funding/scientific-meetings/2011/sex-differences-in-brain-behavior-mental-health-and-mental-disorders/index.shtml.

Nimmerjahn, A. Kirchhoff, F. Helmchen, F.. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science, 308 (2005), pp. 1314–1318

Nixon, K., Kim, D., Potts, E., He, J., & Crews, F. (2008). Distinct cell proliferation events during abstinence after alcohol dependence: Microglia proliferation precedes neurogenesis. *Neurobiology of Disease, 31*(2), 218-229. doi:10.1016/j.nbd.2008.04.009

O'Kusky, J. R., Ye, P., & D'Ercole, A. J. (2000). Insulin-like growth factor-I promotes neurogenesis and synaptogenesis in the hippocampal dentate gyrus during postnatal development. *Journal of Neuroscience.*

Olah, M., Biber, K., Vinet, J., & Boddeke, H. (2011). Microglia Phenotype Diversity. *CNS & Neurological Disorders - Drug Targets (Formerly Current Drug Targets - CNS & Neurological Disorders), 10*(1), 108-118.

Paolicelli, R. C. *et al.* Synaptic pruning by microglia is necessary for normal brain development. *Science* 333, 1456–1458 (2011)

Paula-Barbosa, M., Brandao, F., Madeira, M., & Cadete-Leite, A. (1993). Structural changes in the hippocampal formation after long-term alcohol consumption and withdrawal in the rat. *Addiction, 88*(2), 237-247.

Quandt, J., & Dorovini-Zis, K. (2004). The Beta Chemokines CCL4 and CCL5 Enhance Adhesion of Specific CD4 T Cell Subsets to Human Brain Endothelial Cells. *J Neuropathol Exp Neurol Journal of Neuropathology & Experimental Neurology, 63*(4), 350-362.

Qin, L., He, J., Hanes, R. N., Pluzarev, O., Hong, J. S., & Crews, F. T. (2008). Increased systemic and brain cytokine production and neuroinflammation by endotoxin following ethanol treatment. Journal of Neuroinflammation, 5, 10.

Resnicoff, M., Sell, C., Ambrose, D., Baserga, R., & Rubin, R. (1993). Ethanol inhibits the autophosphorylation of the insulin-like growth factor 1 (IGF-1) receptor and IGF-1-mediated proliferation of 3T3 cells. *Journal of Biological Chemistry, 268*.

Rojdman., R., A., & B. (2000). Insulin-like growth factor (IGF)-1 and IGF-binding protein-1 concentrations in serum of normal subjects after alcohol ingestion: Evidence for decreased IGF-1 bioavailability. *Clinical Endocrinology, 52*(3), 313-318. doi:10.1046/j.1365-2265.2000.00908.x

Shaftel, S. S., Griffin, W. S., & O'banion, M. K. (2008). The role of interleukin-1 in neuroinflammation and Alzheimer disease: An evolving perspective. *Journal of Neuroinflammation J Neuroinflammation, 5*(1), 7. doi:10.1186/1742-2094-5-7

Schultz, C., Temming, P., Bucsky, P., Gopel, W., Strunk, T., & Hartel, C. (2004). Immature anti-inflammatory response in neonates. Clinical and Experimental Immunology Clin Exp Immunol, 135(1), 130-136.

Schwarz, J., & Bilbo, S. (2014). Microglia and Neurodevelopment: Programming of Cognition throughout the Lifespan. The Wiley-Blackwell Handbook of Psychoneuroimmunology Kusnecov/The Wiley-Blackwell, 296-312.

Sierra, A., Encinas, J., Deudero, J., Chancey, J., Enikolopov, G., Overstreet-Wadiche, L., Maletic-Savatic, M. (2010). Microglia Shape Adult Hippocampal Neurogenesis through Apoptosis-Coupled Phagocytosis. *Cell Stem Cell, 7*(4), 483-495.

Stephan, A., Barres, B., & Stevens, B. (2012). The Complement System: An Unexpected Role in Synaptic Pruning During Development and Disease. *Annu. Rev. Neurosci. Annual Review of Neuroscience, 35*, 369-389.

Stratton, K., Howe, C., & Battaglia, F. (1996). Fetal Alcohol Syndrome: Diagnosis, Epidemiology, Prevention, and Treatment. *Washington D.C.: National Academy Press*. doi:10.17226/4991

Streissguth, A., Barr, H., Sampson, P., Darby, B., & Al, E. (1989). IQ at age 4 in relation to maternal alcohol use and smoking during pregnancy. Developmental Psychology, 25(1), 3-11.

Streissguth, A., Barr, H., & Sampson, P. (1990). Moderate Prenatal Alcohol Exposure: Effects on Child IQ and Learning Problems at Age 7 1/2 Years. Alcoholism: Clinical and Experimental Research Alcoholism Clin Exp Res, 14(5), 662-669.

Topper, L. A., Baculis, B. C., & Valenzuela, C. F. (2015). Exposure of neonatal rats to alcohol has differential effects on neuroinflammation and neuronal survival in the cerebellum and hippocampus. *Journal of Neuroinflammation J Neuroinflammation, 12*(1). doi:10.1186/s12974-015-0382-9

Ueno, M., Fujita, Y., Tanaka, T., Nakamura, Y., Kikuta, J., Ishii, M., & Yamashita, T. (2013). Layer V cortical neurons require microglial support for survival during postnatal development. *Nature Neuroscience Nat Neurosci, 16*(5), 543-551. doi:10.1038/nn.3358

Vaurio, L., Riley, E., & Mattson, S. (2011). Neuropsychological Comparison of Children with Heavy Prenatal Alcohol Exposure and an IQ-Matched Comparison Group. Journal of the International Neuropsychological Society J Int Neuropsychol Soc, 17, 463-473.

Wang, C. C., Wu, C. H., SHieh, J. Y., Wen, C. Y., & Ling, E. A. (1996). Immunohistochemical study of amoeboid microglial cells in fetal rat brain. *Journal of Anatomy*.

Xu J, LING EA (1994a) Expression of major histocompatibility complex class II antigen on amoeboid microglial cells in early postnatal rat following intraperitoneal injections of lipopolysaccharide. Experimental Brain Research 100, 287-292.

Xu J, LING EA (1994b) Upregulation and induction of surface antigens with special reference to MHC class II expression on microglia in postnatal rat brain following intravenous or intraperitoneal injections of lipopolysaccharide. Journal of Anatomy 184, 285-296.

Xu J, LING EA (1994c) Upregulation and induction of MHC class I and II antigens on microglial cells in early postnatal rat brain following intraperitoneal injections of recombinant interferongamma. Neuroscience 60, 959-967.

Yang, J., Xue, X., Tian, H., & Wang, X. (2014). Role of microglia in ethanol-induced neurodegenerative disease: Pathological and behavioral dysfunction at different developmental stages. *Pharmacology & Therapeutics, 144*(3), 321-37.

Yirmiya, R., & Goshen, I. (2011). Immune modulation of learning, memory, neural plasticity and neurogenesis. *Brain, Behavior, and Immunity, 25*(2), 181-213. doi:10.1016/j.bbi.2010.10.015

Zhao, Y., Wang, F., Fan, Y., Ping, G., Yang, J., & Wu, C. (2013). Activated microglia are implicated in cognitive deficits, neuronal death, and successful recovery following intermittent ethanol exposure. *Behavioural Brain Research, 236*, 270-282. doi:10.1016/j.bbr.2012.08.052

Zhou, D., Lebel, C., Lepage, C., Rasmussen, C., Evans, A., Wyper, K., . . . Beaulieu, C. (2011). Developmental cortical thinning in fetal alcohol spectrum disorders. NeuroImage, 58(1), 16-25.

Zimmerman, H. J. (1978). *Hepatotoxicity: The adverse effects of drugs and other chemicals on the liver*. New York: Appleton-Century-Crofts.

APPENDIX A

Animal Protocol Permissions

A.1 INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE ANNUAL REVIEW

University of Delaware
Institutional Animal Care and Use Committee
Annual Review

RECEIVED
MAY 7 2015
IACUC JH

(Please complete below using Arial, size 12 Font.)

Title of Protocol: Therapeutic Motor Training and Fetal Alcohol Effects

AUP Number: 1134-2015-2 ← **(4 digits only)**

Principal Investigator: Dr. Anna Y. Klintsova

Common Name: Rat, Long-Evans

Genus Species: Rattus norvegiccus

Pain Category: ***(please mark one)***

USDA PAIN CATEGORY: ***(Note change of categories from previous form)***

Category	Description
☐ B	Breeding or holding where NO research is conducted
☐ C	Procedure involving momentary or no pain or distress
X D	Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)
☐ E	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

Official Use Only

IACUC Approval Signature: Glenn Tallia, DVM

Date of Approval: 7-1-15

Principal Investigator Assurance

1. I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.
2. I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (OLAW).
3. I understand that the Attending Veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.
4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist listed on this AUP. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.
5. I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.
6. I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.
7. I assure that any modifications to the protocol will be submitted to the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.
8. I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.
9. I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.
10. I assure that the experimental design has been developed with **consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.**
11. I assure that the proposed research does not unnecessarily duplicate previous experiments. *(Teaching Protocols Exempt)*
12. I understand that by signing, I agree to these assurances. A Klontsova — May 07, 2015 Signature of Principal Investigator — Date

SIGNATURE(S) OF ALL PERSONS LISTED ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only the procedures that have been approved by the IACUC.

Name	Signature
1. Anna Klintsova	
2. Karen Boschen	
3. Kerry Criss	
4. Zachary Gursky	
5. Sarah McKeown	
6. Michael Ruggiero	
7. Zubin Hussain	
8. Shaqran Shareeq	
9.	
10.	
11.	
12.	
13.	
14.	
15.	

IACUC approval of animal protocols must be renewed on an annual basis.

1. **Previous Approval Date:** July 1, 2014

   Is Funding Source the same as on original, approved AUP?

   X Yes ☐ **No**

   If no, please state Funding Source and Award Number:

2. **Record of Animal Use:**

Common Name	Genus Species	Total Number Previously Approved	Number Used To Date
1. Rat (Long Evans)	Rattus norvegicus	504 (+300, pending approval)	594
2.			
3.			
4.			
5.			

3. **Protocol Status:** *(Please indicate by check mark the status of project.)*

   Request for Protocol Continuance:

   X A. Active: Project ongoing

   ☐ B. Currently inactive: Project was initiated but is presently inactive

   ☐ C. Inactive: Project never initiated but anticipated starting date is:

   Request for Protocol Termination:

   ☐ D. Inactive: Project never initiated

   ☐ E. Completed: No further activities with animals will be done.

4. **Project Personnel:** Have there been any personnel changes since the last IACUC approval? X Yes ☐ No

   If Yes, fill out the Amendment to Add/Delete Personnel form to "Add" Personnel.

Project Personnel Deletions:

Name	Effective Date
1. Sarah McKeown	June 1, 2015
2.	
3.	
4.	
5.	

5. Progress Report: If the status of this project is 3.A or 3.B, please provide a brief update on the progress made in achieving the aims of the protocol. We have completed tissue collection for ELISA protein analysis (BDNF and TrkB) for PD10 as described in the **3-year Animal Protocol Renewal (2013) Experimental Design**. We need to generate 2 more cohorts of experimental animals (8 litters in each, 4 suckle control litters and 4 intubated, approximately 96 animals) to evaluate apoptosis, cell proliferation and neurogenesis at PD10 time point. We generated 2 more cohorts of alcohol-exposed animals for the wheel running(WR)-environmental complexity(EC) study (8 more litters, 12-14 pups in each litter (106 animals total) that are being used in neuroanatomical study (evaluation of adult neurogenesis; neurotrophic factors expression in prefrontal cortex and hippocampus). In addition, we generated 2 cohorts of alcohol-exposed animals [8 litters x 8 pups (after culling) = 64 rats] that were used for protein and mRNA estimation after WR/EC at PD72.

6. **Problems or Adverse Effects:** If the status of this project is 3.A or 3.B, please describe any unanticipated adverse events, morbidity, or mortality, the cause if known, and how these problems were resolved. If there were none, this should be indicated.

No unanticipated adverse events in terms of animal welfare happened.