ALTERATIONS TO HIPPOCAMPAL NEUROPLASTICITY ACROSS THE LIFESPAN IN A RODENT MODEL OF FASD

by

Karen E. Boschen

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Psychology

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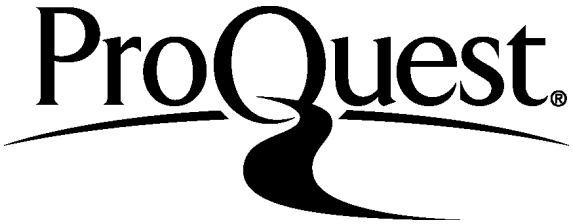

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Karen E. Boschen

Approved: _____

Robert Simons, Ph.D.
Chair of the Department of Psychology

Approved: _____

George H. Watson, Ph.D.
Dean of the College of Arts & Sciences

Approved: _____

Ann L. Ardis, Ph.D.
Senior Vice Provost for Graduate and Professional Education

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Anna Y. Klintsova, Ph.D.
Professor in charge of dissertation

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Tania L. Roth, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Jeffrey B. Rosen, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Jaclyn M. Schwarz, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Alexandre E. Medina, Ph.D.
Member of dissertation committee

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LIST OF ABBREVIATIONS

AE	alcohol-exposed
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
BAC	blood alcohol concentration
BDNF	brain-derived neurotrophic factor
BrdU	bromodeoxyuridine
C	cytosine
CA	cornus ammonis
CCL	C-C motif chemokine ligand
CD11b	cluster of differentiation 11b
CREB	cyclic-AMP response-element-binding
DAB	diaminobenzidine
DCX	doublecortin
DG	dentate gyrus
DNA	deoxyribonucleic acid
E	embryonic day
EC	environmental complexity
FAS	fetal alcohol syndrome
FASD	fetal alcohol spectrum disorders
g	gram
G	guanine

GABA	gamma-aminobutyric acid
h	hour(s)
HDACs	histone deacetylases
Iba-1	ionized calcium-binding adapter molecule 1
IGF	insulin-like growth factor
IL	interleukin
i.p.	intraperitoneal
kg	kilogram
LPS	lipopolysaccharide
LTP	long-term potentiation
MeCP2	Methyl-CpG Binding Protein 2
µm	micrometer
mg	milligram
min	minutes
ml	milliliter
mM	millimolar
mPFC	medial prefrontal cortex
mRNA	messenger ribonucleic acid
msec	milliseconds
MSP	methylation-specific polymerase chain reaction
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
ng	nanograms

NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NT	neurotrophin
PB	phosphate buffer
PD	postnatal day
PFC	prefrontal cortex
RNA	ribonucleic acid
rpm	revolutions per minute
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
SC	suckle control
SD	standard deviation
SEM	standard error of the mean
SH	standard social housing
SI	sham intubated
TBS	tris buffer solution
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α
VIP	vasoactive intestinal peptide
v/v	volume/volume
VEGF	vascular endothelial growth factor
WR	wheel running

ABSTRACT

Alcohol exposure *in utero* can result in Fetal Alcohol Spectrums Disorders (FASD) in the child. Patients with FASD exhibit permanent structural changes in various brain regions, including the hippocampus and functional impairments in many aspects of cognition and behavior. Measures of hippocampal neuroplasticity, including long-term potentiation, synaptic and dendritic organization, and adult neurogenesis, are consistently disrupted in rodent models of FASD. Alterations to these neuroplastic processes contribute to learning and memory deficits observed in individuals with prenatal alcohol exposure. This dissertation examines brain-derived neurotrophic factor (BDNF) as a molecule potentially altered following neonatal alcohol exposure in the rat hippocampus and the neuroimmune response as a potential pathway that exacerbates alcohol-induced damage. BDNF is critical for cell proliferation, maturation, and dendritic outgrowth in the developing and adult brain. Previous work from our lab demonstrated that the number of adult-born dentate gyrus granule cells surviving to maturation is impaired in neonatal alcohol-exposed rats (Klintsova et al., 2007; Hamilton et al., 2012). This dissertation utilizes a third trimester-equivalent (postnatal day [PD] 4-9) binge-like alcohol exposure rat model (AE; 5.25 g/kg/day) with two control groups, sham-intubated (SI) and undisturbed/suckle control (SC). Specific Aim 1 investigated how the brain initially responds to binge-like alcohol exposure by analyzing short-term alterations to expression of BDNF and the neuroimmune response in the hippocampus. Experiment 1 examined protein levels of BDNF and its high-affinity receptor TrkB, *Bdnf* exon-specific gene expression, and

Bdnf DNA methylation in neonatal animals 24 hours following AE (PD10). Experiment 2 analyzed microglial number and activation state, as well gene expression of pro- and anti-inflammatory cytokines on PD10. For Experiment 1, BDNF protein and total gene expression levels were increased in AE animals at PD10, with increases also being found in the sham-intubated animals. Alcohol-specific upregulation of TrkB and *Bdnf* exon I-specific gene expression was also found. *Bdnf* exon I DNA methylation was decreased in the AE and SI animals. For Experiment 2, there were fewer microglia in the dentate gyrus and CA1 of AE and SI animals, increased microglia activation in CA1, DG and CA3 specific to the AE group as measured by cell territory, and increased IL-1β, TNF-α, CCL4 and CD11b gene expression in AE and SI animals. CCL4 was even further elevated in AE animals compared to SI. Levels of anti-inflammatory cytokine TGF-β were increased in AE animals compared to SI and SC. This Aim supports investigation of BDNF and TGF-β as neuroprotective responses to alcohol-related insult. Specific Aim 2 examined the long-term effects of neonatal alcohol exposure on BDNF protein and exon-specific gene expression and *Bdnf* DNA methylation in the hippocampus of adult rats (PD72). In addition, animals were exposed to either a "super-intervention" (WREC; voluntary wheel running (PD30-42) followed by housing in a complex environment (PD42-72)) or continuous wheel running (WRWR; PD30-72). Both interventions have been shown to enhance BDNF and adult neurogenesis (Vivar et al., 2013; Olson et al., 2006; Hamilton et al., 2012). Alterations to basal BDNF protein and gene expression levels were transient as AE animals do not differ from controls on PD72 in standard housing.

Rats housed in WRWR, but not WREC, show higher levels of hippocampal BDNF protein and gene expression. Steady-state *Bdnf* exon I methylation was not altered by neonatal condition, however, WRWR was associated with less methylation in control animals. Specific Aim 3 investigated whether AE affects hippocampal adult neurogenesis and dendritic morphology in adult (PD72) male rats and whether exposure to either WREC or WRWR impacts these measures. As expected, AE did not affect cell proliferation or number of immature neurons, but significantly decreased dendritic complexity of the immature neurons compared to SI and SC groups. Specifically, there were significant decreases in dendritic material, number of intersections, and number of bifurcations per radius. WREC and WRWR both robustly enhanced dendritic complexity in the AE animals, and while WRWR modestly increased complexity in SI and SC animals and WREC had little effect. Specific Aims 3 demonstrates the long-lasting impact of neonatal alcohol exposure on dendritic morphology of immature neurons in the hippocampus, which could contribute to alcohol-related deficits in granule cell survival observed in this model. These studies also support the implementation of exercise and complex environments as therapeutic interventions for individuals with FASD.

Chapter 1

INTRODUCTION

Even more than the muscles in our bodies, the brain can be strengthened, shaped, and reshaped in incredible and intricate ways to adapt to the evolving world around us. Musculature and the brain both respond to use and experience, though, by comparison, brain plasticity is a much more delicate and complex process. This knowledge has opened new therapeutic options to patients with debilitating brain injuries and helped scientists understand how we learn, form new memories, and develop and recover from neurological disorders and psychopathologies. However, the notion that the brain was plastic and malleable was not always accepted. In fact, as recently as the 1970s, neuroscientists resisted the idea that the brain can change itself, either in order to create new memories or to recover from trauma. However, the insight that changes to brain structure and function must be a necessity for an organism to adapt to changing environmental needs has been discussed for over a century. Ramon y Cajal proposed changes to synaptic structure as a requirement for new learning as early as 1894, and a mechanism for these changes, later referred to as Hebbian theory, was hypothesized by Donald Hebb in the 1940's (Hebb, 1949). After decades of clinical observation and basic research, the idea that the brain can reorganize and respond to external stimuli is an idea that is not only accepted, it is embraced.

1.1 Brief Introduction to Neuroplasticity in the Hippocampus

The term neuroplasticity can signify both structural and functional changes in the brain. Synaptic plasticity refers to changes that affect how neurons communicate with one another, such as the addition or deletion of receptors from a synapse which can ease or inhibit cell communication (Citri & Malenka, 2008). Non-synaptic plasticity also occurs, such as the growth or retraction of dendrites and dendritic spines or the generation of new neurons and glial cells. While any brain region can undergo neuroplastic changes, areas involved in learning and memory have been studied in depth. One region in particular, the hippocampus, has been well-characterized for its role in spatial and contextual learning and plays a prominent role in the neuroplasticity literature (Andersen et al., 2007).

The hippocampus is necessary for the formation of spatial memories and contextual representations. In the rodent, the hippocampus is dorsally located and easily accessed with electrodes to record both population and individual neuronal firing patterns. Many pyramidal neurons in the CA1 subregion of the hippocampus exhibit spatially-dependent firing patterns (called “place cells”) which evolve as an animal learns a new context (O’Keefe & Dostrovsky, 1971). The long-term potentiation of synaptic communication, which depends on the insertion of additional AMPA receptors into the postsynaptic membrane and subsequent protein synthesis, has been repeatedly and robustly demonstrated in the hippocampus (Bliss & Collingridge, 1993). One region of the hippocampus, the dentate gyrus is one of two brain regions known to continually produce new cells throughout the lifespan

(Kempermann, 2011). These new cells can develop into fully functioning and integrated granule cells and are thought to play an important role in new task acquisition and pattern separation. These features of the hippocampus make it inherently suitable to foster neuroplastic alterations and respond robustly to environmental stimulation.

1.2 Brief Introduction Fetal Alcohol Spectrum Disorders and Hippocampal Dysfunction

The potential for plasticity in the developing brain can be greatly impaired by exposure to teratogenic agents, such as alcohol. Fetal Alcohol Spectrum Disorders (FASD) is an umbrella term referring to a variety of physical, cognitive, and behavioral effects caused by prenatal exposure to alcohol and is estimated to affect up to 5% of live births each year in the United States (May et al., 2009; Morleo et al., 2011; Sampson et al., 1997). The most severe form of FASD is Fetal Alcohol Syndrome (FAS), which is characterized by craniofacial dysmorphologies, low birth and brain weight, motor impairments, and long-lasting neurological and behavioral deficits (Mattson et al., 1996; Mattson et al., 1997; Mattson et al., 2001) Children with FASD show executive functioning (e.g. impaired judgment and cognitive flexibility, increased risk-taking behavior) and spatial memory impairments (Mattson et al., 1999). These deficits are likely due to the damaging effect of alcohol on brain structures such as the hippocampus, prefrontal cortex, corpus callosum, cerebellum, and basal ganglia (Archibald et al., 2001; Autti-Ramo et al., 2002; Mattson et al., 1996; Riley et al., 1995). Much of our understanding of how alcohol affects the

developing brain comes from work in animal models, which have allowed researchers to determine the impact of alcohol dose and timing of exposure. For example, alcohol exposure during the first trimester of exposure, particularly during gastrulation and neurulation, can affect the closing of the neural tube and development of midline structures, resulting in the craniofacial abnormalities classically associated with FAS. In rodents, the human third trimester-equivalent of brain development takes places during the first two postnatal weeks (Dobbing & Sands, 1979), with significant neurogenesis, synaptogenesis, and gliogenesis still taking place in late-developing structures such as the hippocampus, prefrontal cortex, and cerebellum (Crocker et al., 2011; Kooistra et al., 2010; Mattson et al., 2001; Mattson et al., 2011). Alcohol exposure during the third trimester-equivalent causes severe and long-lasting damage in these structures (Klintsova et al., 2002; Klintsova et al., 2007; Thomas et al., 2008; Gil-Mohapel et al., 2011; Hamilton et al., 2015).

Developmental alcohol exposure impairs multiple measures of neuroplasticity in the hippocampus, which likely contributes to alcohol-induced deficits in learning and memory. Studies have demonstrated that third trimester-equivalent alcohol exposure disrupts hippocampal long-term potentiation (Puglia & Valenzuela et al., 2010 a, b), expression of neuronal activity marker c-Fos (Clements et al., 2005; Murawski et al., 2012), adult neurogenesis (Klintsova et al., 2007; Hamilton et al., 2012), decreases CA1 pyramidal cell number (Bonthius & West, 1990, 1991), and impairs performance on hippocampal-associated behavioral tasks (Berman & Hannigan, 2000; Hamilton et al., 2014). These long-term deficits arise from damage

taking place during or shortly following the alcohol exposure. Even single exposures to alcohol cause widespread apoptosis throughout the developing brain through "silencing" of neuronal activity during this sensitive and critical period (Ikonomidou et al., 2000; Olney et al., 2002; Farber et al., 2010; Lebedeva et al., 2015).

1.3 Brief Introduction to Behavioral Interventions and Neuroplasticity in the Hippocampus

Discovery and advancement of therapeutic interventions to reverse the detrimental effects of alcohol exposure during brain development is an important research area, as conventional public awareness campaigns have plateaued in their effectiveness to decrease maternal drinking rates and combat misconceptions regarding the dangers of drinking during pregnancy. Two behavioral interventions with strong translational possibilities are aerobic exercise and exposure to a complex environment. In rodent models, aerobic exercise is usually achieved through voluntary running on a wheel or through forced walking or running on a treadmill. Environmental complexity paradigms can vary widely between laboratories, but most include an aspect of increased opportunity for social interaction and enhanced cognitive stimulation through access to novel toys and objects. Some models also include access to a running wheel as part of the intervention. Both exercise and environmental complexity enhance neuroplasticity and behavioral outcomes (van Praag et al., 1999; Lucas et al., 2012; Mustroph et al., 2012; Olson et al., 2006; Angevaren et al., 2008; Maniam & Morris, 2010). One way in which these interventions affect neuroplasticity is through the upregulation of neurotrophic factors,

including brain-derived neurotrophic factor (BDNF) (Rasmussen et al., 2009; Griffin et al., 2009; Gomes da Silva et al., 2012; Marlatt et al., 2012). This neurotrophin is critical for cell maturation processes, induction of long-term potentiation, dendritic outgrowth, and memory formation. Furthermore, BDNF has anti-apoptotic effects through action at its high-affinity receptor, tropomyosin-related kinase B (TrkB) (Climent et al., 2002; Almeida et al., 2005; Han et al., 2000). Thus, not only has BDNF been shown to be enhanced by behavioral interventions and have therapeutic potential, expression of this neurotrophin could be dysregulated following alcohol exposure in the developing brain.

1.4 Dissertation Questions

This dissertation examines hippocampal neuroplasticity in a rat model of FASD. Specifically, I investigate whether developmental alcohol exposure from postnatal days 4-9 impacts the short-term expression and DNA methylation of BDNF and microglial activation in the neonatal hippocampus. I also examine whether neonatal alcohol exposure affects the long-term expression of BDNF and the process of adult neurogenesis in the adult rat hippocampus. Chapter 4 discusses previously published work which provides the foundation for the dissertation studies presented here. I discuss in detail the work from our laboratory and other research groups showing that 1) developmental alcohol exposure impacts short- and long-term BDNF synthesis, 2) neonatal alcohol exposure induces a neuroimmune response, and 3) exercise and environmental complexity enhance neuroplasticity in the adult rat hippocampus.

Rodent models of FASD have demonstrated that developmental alcohol exposure alters BDNF expression in the neonatal hippocampus, though these alterations are highly dependent on timing of the exposure and alcohol dose (Heaton et al., 2000; Heaton et al., 2003; Light et al., 2001; Fattori et al., 2008; Caldwell et al., 2008; Boehme et al., 2011; Ceccanti et al., 2012; Miki et al 2008). In particular, I was intrigued by literature showing that neonatal alcohol exposure via vapor inhalation upregulated hippocampus BDNF and NGF expression (Heaton et al., 2003) less than 24 hours following the exposure. In addition, there was emerging evidence that neonatal alcohol exposure could induce a neuroinflammatory response in the developing brain through production of pro-inflammatory cytokines (Tiwari & Chopra, 2011; Kane et al., 2011; Drew et al., 2015; Topper et al., 2015). Taken together, these alterations to neurotrophin levels and the neuroimmune response, depending on their directionality, could represent either a secondary source of damage or a compensatory neuroprotective response following neonatal alcohol exposure. Thus, two questions were asked in Specific Aim 1: 1) *does neonatal alcohol exposure (PD4-9) alter BDNF and TrkB protein expression, Bdnf gene expression, and Bdnf DNA methylation in the neonatal hippocampus*, and 2) *does neonatal alcohol exposure impact microglial cell number and morphology or the production of pro- and anti-inflammatory cytokines in the neonatal hippocampus?*

The literature regarding whether developmental alcohol exposure affects the expression of BDNF in the adult hippocampus is inconsistent due to the various experimental paradigms used. The negative impact of third trimester-equivalent

alcohol exposure on measures of hippocampal neuroplasticity, however, is a consistent finding, supporting that alterations to BDNF synthesis, trafficking, or release are likely and contribute to the observed deficits in plasticity. For Aim 2 of this dissertation, the goal was to extend some of the findings from Aim 1 using a lifespan approach and investigate these measures in the adult (postnatal day 72) hippocampus. Specifically, I asked *does neonatal alcohol exposure (PD4-9) alter Bdnf exon-specific gene expression and DNA methylation in the adult rat hippocampus?* The impact of two behavioral interventions, exercise (voluntary wheel running) and housing in complex environment, on BDNF synthesis in the adult hippocampus was also of paramount interest for this dissertation. Both of these interventions have been shown to enhance neuroplasticity and BDNF levels in the healthy and damaged brain (van Praag et al., 1999; Lucas et al., 2012; Mustroph et al., 2012; Olson et al., 2006; Rasmussen et al., 2009; Griffin et al., 2011; Gomes de Silva et l., 2012; Marlatt et al., 2012). Thus, I asked *does housing with access to wheel running or with access to wheel running followed by housing in a complex environment impact Bdnf exon-specific gene expression and DNA methylation in the adult hippocampus of neonatally alcohol-exposed or control rats?*

The final Aim of my dissertation focused on extending previous work from our laboratory investigating how third trimester-equivalent (postnatal day 4-9) alcohol exposure affects the process of adult neurogenesis and dendritic morphology of immature dentate gyrus granule cells in the postnatal day 72 rat hippocampus. Previous work from our research group has shown that our model of FASD spares the

ability of the hippocampus to generate new neurons but impairs the survival rate of these adult-born cells (Klintsova et al., 2007; Hamilton et al., 2012), potentially contributing to disrupted behavioral performance on hippocampal-associated tasks. My dissertation focused on cell proliferation and the number and morphology of immature neurons (labeled with the marker doublecortin [DCX]) in the adult rat hippocampus as changes to these parameters could be indicative of alcohol-related damage and impact the number of adult-generated mature neurons. Specifically, I asked *does neonatal alcohol exposure (PD4-9) alter the number of proliferating cells (Ki-67+), the number of immature neurons (DCX+), or the dendritic morphology of the DCX+ neurons in the adult rat hippocampus?* Exercise followed by environmental complexity rescues the survival of adult-born granule cells in our model of FASD (Hamilton et al., 2012; Hamilton et al., 2014), and these interventions increase dendritic complexity in various cell type throughout the brain (Redila & Christie, 2006; Eadie et al., 2005; Hamilton et al., 2015). Thus, the second question I asked in Specific Aim 3 was *does housing with access to wheel running or with access to wheel running followed by housing in a complex environment impact the number of proliferating cells (Ki-67+), the number of immature neurons (DCX+), or the dendritic morphology of the DCX+ neurons in the adult dentate gyrus of neonatally alcohol-exposed or control rats?*

1.5 Dissertation Summary

This dissertation is divided into three main components. Part 1 (Chapters 2-5) discuss relevant background information, including a detailed introduction to the

neurobiology of Fetal Alcohol Spectrum Disorders, the hippocampus, brain-derived neurotrophic factor (BDNF), and the behavioral interventions of exercise and environmental complexity. Chapter 4 (Foundational Studies) describes the most relevant literature which supports the importance and validity of the questions asked in this dissertation, including previous work investigating the impact of alcohol exposure on BDNF, the neuroimmune response, adult neurogenesis, and dendritic complexity. In addition, it discusses work detailing how exercise and environmental complexity affect BDNF production, new cell proliferation and survival, and dendritic morphology. Chapter 5 gives an introduction to the experimental methods and techniques utilized in the three Specific Aims. Part 2 of the dissertation (Chapter 6-8) describes the rationale, methodology, and results for each of my Specific Aims. In Chapter 6, I discuss my findings regarding whether neonatal alcohol exposure affects BDNF synthesis and neuroinflammation in the developing rat hippocampus (Specific Aim 1). Chapters 7 and 8 focus on my findings regarding BDNF (Specific Aim 2) and adult neurogenesis and dendritic morphology (Specific Aim 3) in the adult rat hippocampus following neonatal alcohol exposure. The influence of exercise and environmental complexity on these measures is also reported and discussed in these chapters. Finally, Part 3 of the dissertation (Chapter 9) summarizes my findings, discusses conclusions drawn from this work and any experimental limitations, and considers future research directions.

Chapter 2

FETAL ALCOHOL SPECTRUM DISORDERS (FASD) AND THE HIPPOCAMPUS

During prenatal development, the fetus could be exposed to a variety of environmental or infectious agents known as teratogens which disturb normal growth and survival, ultimately resulting in malformations to the fetus or "birth defects." Common teratogens include pesticides, infections, drugs of abuse, and prescription medication. Teratogens exert their deleterious effects through a variety of pathways and the resulting defects can range from severe (limb loss following maternal use of thalidomide) to subtle (delayed language skills following prenatal cocaine exposure). One of the most widely used teratogenic agents is alcohol, which will be the focus of this chapter[1].

Alcohol (ethyl alcohol or ethanol, specifically) is a commonly used psychoactive drug with sedative properties. Primarily used recreationally, over 70% of adults over age 18 report using alcohol in the past year (National Institute of Alcohol and Alcoholism, 2016). Binge drinking (usually defined as >4 drinks in one sitting) is also prevalent, with over 24% of adults reporting at least one episode of binge drinking in the past month. Alcohol exerts its sedative effects through inhibition of NMDA-subtype glutamate receptor activity and enhancement of chloride ion flow

[1] Text and figures used in this chapter have been reproduced or adapted with permission from Klintsova, Hamilton & Boschen (2013) and Boschen & Klintsova, *in press.*

through $GABA_A$ receptor ion channels. In adults, even low doses of alcohol can result in impaired motor control, judgment and reaction time due to the widespread and potent effects of alcohol on multiple brain regions.

Pregnant women also report relatively high rates of alcohol consumption, with 20-30% of women reporting some degree of alcohol use during their pregnancy, despite well-publicized campaigns to warn women of potential danger to the developing fetus. When a pregnant woman ingests alcohol, the alcohol easily passes through the placenta, meaning that blood alcohol concentrations in the fetus are comparable to or even higher than those recorded from the mother (Nava-O'Campo et al., 2004; van Faassen & Niemelä, 2011). Alcohol is easily metabolized in adults through the action of alcohol dehydrogenase in the stomach and liver; however, fetuses lack the enzymes necessary for the breakdown of alcohol. Thus, the alcohol is only metabolized after being circulated back into the maternal blood supply, meaning that concentrations of alcohol within the placenta could be higher than the mother's blood alcohol concentration and remain elevated for a prolonged period (van Faassen & Niemelä, 2011).

2.1 Fetal Alcohol Spectrum Disorders (FASD)

Fetal exposure to alcohol can cause a wide range of long-lasting physiological and behavioral effects, collectively referred to as Fetal Alcohol Spectrum Disorders (FASD). The most severe form of fetal alcohol effects is Fetal Alcohol Syndrome (FAS), which manifests with craniofacial malformations, including small eyes and palpebral fissure, smooth philtrum, and a thin upper lip, reduced body and brain

weight, intellectual disabilities, and cognitive and behavioral impairments. Prevalence statistics for the United States have been historically difficult to obtain due to the stigma associated with maternal drinking, making pregnant women less likely to accurately report rates of alcohol consumption. The current statistics estimate that up to 5% of live births each year in the Unites States are affected by FASD, while FAS occurs in approximately 0.1% of live births (Center for Disease Control, 2015; May et al., 2009). Children with FASD, including FAS, can require lifelong care, both in terms of extra medical expenditures in childhood (Amendah et al., 2011) and access to specialized services to help cope with learning disabilities and other behavioral needs. Estimates from 2002 indicated that care for children with FAS costs on average $2 million across the lifespan, and that services for these individuals costs the United States approximately $4 billion a year (1998 estimate; Lupton et al., 2004). More recent estimates or costs including the full range of FASD are not readily available, but it can be inferred that care for these individuals is a significant use of resources, both for families and society as a whole.

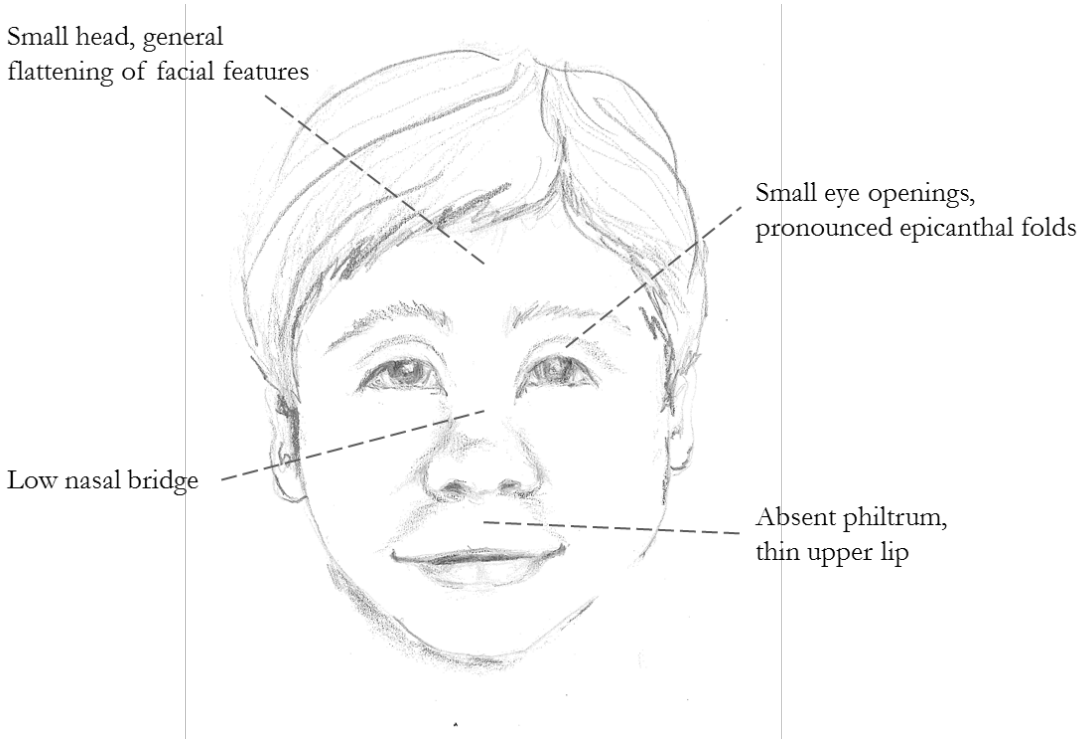

Figure 2.1. Classic Craniofacial Dysmorphologies Associated with Fetal Alcohol Syndrome (FAS). Illustration by P.T. Boschen. Reproduced with permission from Boschen & Klintsova, *in press*.

Cost estimates for individuals with FAS and the full spectrum of FASDs are difficult to calculate due to the range of primary and secondary disabilities caused by prenatal alcohol exposure. Primary disabilities in children with FASDs refer to changes to the brain that directly result in impaired mental function, such as decreased volume of both white and grey matter structures which cause deficient performance on reading, writing and arithmetic tasks (Mattson et al., 1996a, b;). These children often perform poorly on working memory and IQ tests (Mattson et al., 1999; Rasmussen et al., 2011; Mattson et al., 1997) and are more likely to require special education services. These disabilities often extend beyond scholastic performance and can affect

social interactions (Irner et al., 2012; Stevens et al., 2012), executive functioning (Pei et al., 2011), impulse control (Franklin et al., 2008), and emotion regulation (Kodituwakku et al., 1995), often resulting in higher rates of incarceration and mental illness in adulthood (Streissguth et al, 2004).

Public safety campaigns, including the announcement from the American Academy of Pediatrics in 2015 officially recommending no alcohol use during pregnancy, have ultimately not eradicated the existence of a completely preventable cause of mental retardation, leading researchers to continue to investigate 1) the molecular, cellular and system-wide damage caused by prenatal alcohol exposure, and 2) potential behavioral and pharmacological interventions to reverse the detrimental cognitive and behavioral effects. This pursuit has led to a vast number of animal models of FASDs which have been critical in understanding how the dose, timing and pattern of alcohol exposure can impact behavioral and neuroanatomical outcomes.

2.2 Modeling Developmental Alcohol Effects in Experimental Animals

Rats and mice are the most commonly used for FASD models, though sheep and primates are sometimes used as well. Rat and mouse pups are born at a developmentally earlier stage than humans, meaning that brain development occurring during the third trimester of human pregnancy takes place over the first two postnatal weeks in rodents (Dobbing & Sands, 1979). Alcohol exposure during specific points in development results in damage to whatever developmental process is occurring during this time. For example, administration of alcohol during the first trimester-equivalent (gastrulation or neurulation) would cause craniofacial dysmorphologies

analogous to those seen in children with FAS (Sulik, 2005; Lipinski et al., 2012), but administration during the third trimester-equivalent would cause more subtle neuroanatomical damage to late-developing brain structures such as the hippocampus, prefrontal cortex or cerebellum. As mentioned above, neurotrophins also display developmentally-regulated expression patterns, suggesting that interactions between alcohol and neurotrophin function would likely depend on developmental stage.

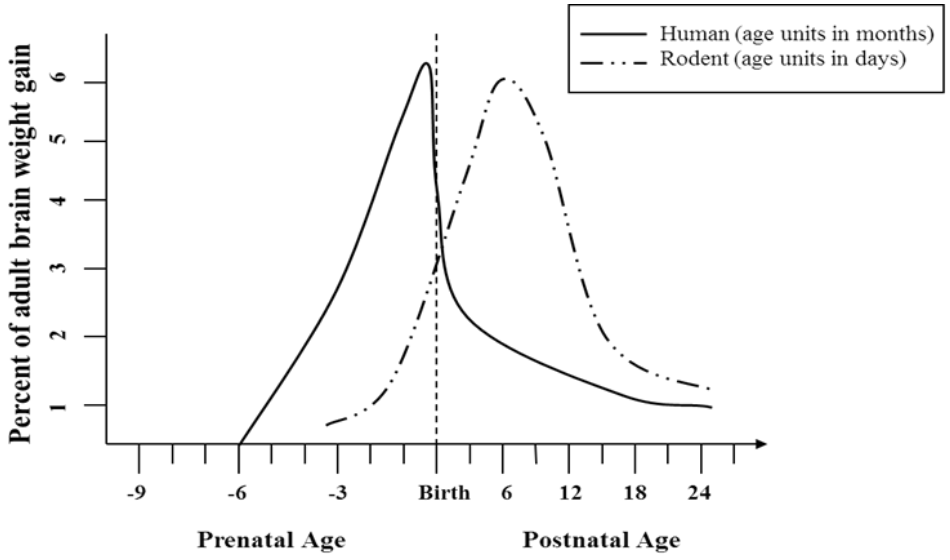

Figure 2.2. Timeline of Comparative Brain Development in Humans and Rodents. Reproduced with permission from Klintsova, Hamilton & Boschen (2013).

Rodent FASD models can be split into two broad categories: prenatal and postnatal exposure. Prenatal models mimic alcohol exposure during approximately the first two trimesters of human pregnancy, while postnatal models mimic exposure during the third trimester (reviewed in Gil-Mohapel et al., 2010). In prenatal models, alcohol is administered to the dam either through maternal voluntary drinking (Figure 2.3A), intraperitoneal or subcutaneous injection, or intragastric gavage. Voluntary

drinking paradigms are relatively low stress for the dam, however, they usually model low to moderate levels of drinking due to the amount of alcohol being ingested is under the control of the dam rather than the experimenter directly. In contrast, injections and gavage are able to achieve higher blood alcohol concentrations (BACs) and the dose of alcohol administered is constant across animals; however, injections and gavage are inherently stressful procedures. Postnatal models deliver the alcohol directly to the pups, enabling control and alcohol-exposed pups to be assigned to the same litter, which leaves maternal care of the pups unaffected. Intragastric intubation (Figure 2.3B) and injections are commonly used methods of alcohol delivery for neonatal pups, achieving high BACs in a controlled dose, with the negative consequence of increased stress and morbidity rate. Vapor inhalation paradigms (Figure 2.3C) are also commonly used, with pups being placed in a chamber filled with ethanol vapor for 1-4 hours at a time. Long periods of maternal separation can be detrimental to pup development, so dams are often placed in the vapor chamber with the pups. As a result, this method usually models low to moderate levels of alcohol exposure in order to leave the dam unimpaired and able to sufficiently care for the pups. While chronic exposure to ethanol vapor can cause mild irritation to mucous membranes, the stress induced by handling during this method is minimal. Two important differences distinguish these FASD models: developmental time point (trimester equivalent) and level of BAC achieved. These two variables can determine the type and severity of alcohol-induced damage depending on what developmental processes are occurring. For instance, alcohol exposure during gastrulation can affect

neural tube closure, while alcohol exposure during the third trimester-equivalent could impact pyramidal cell migration in the prefrontal cortex and development of the hippocampus.

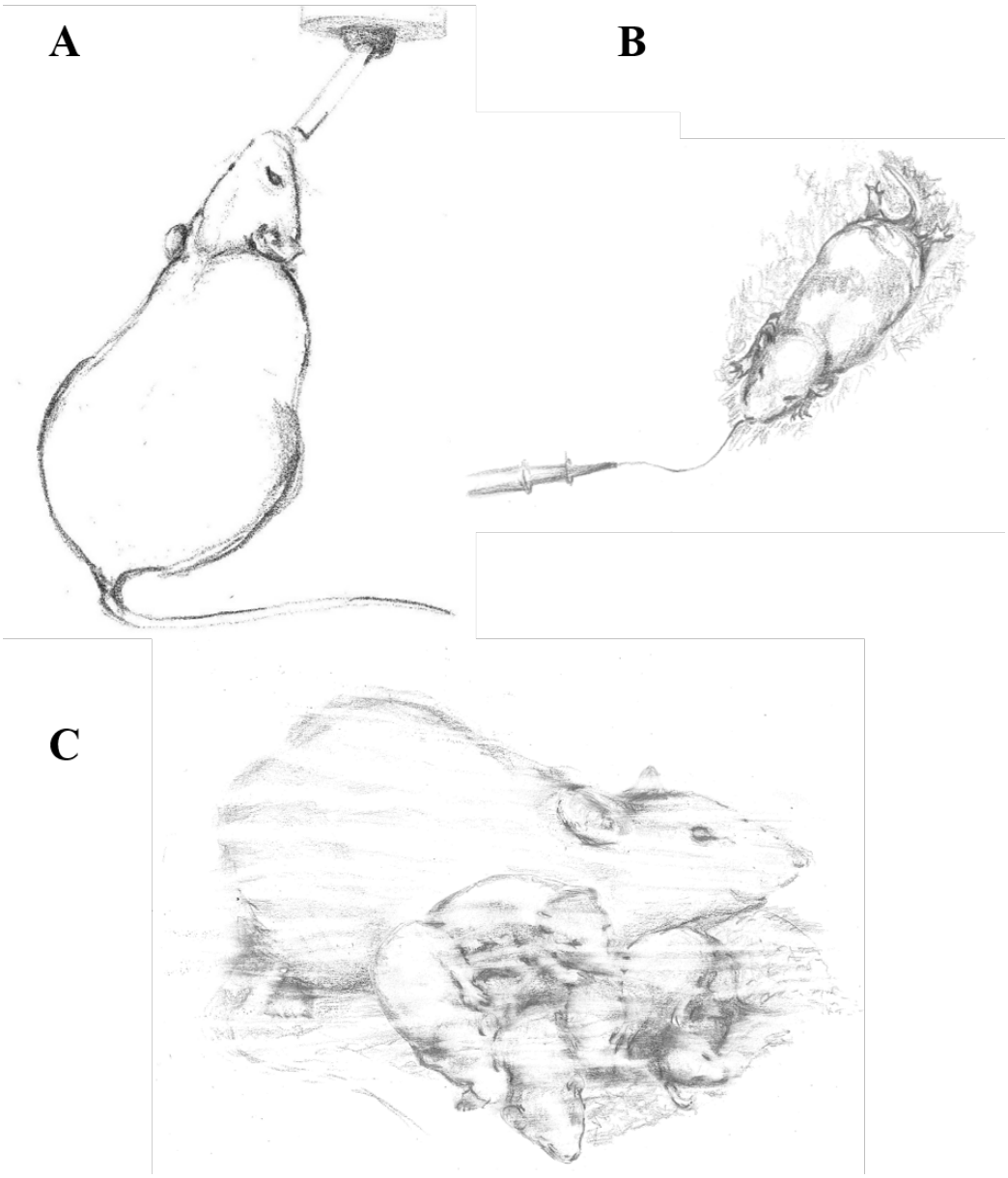

Figure 2.3. Representative Illustration of Alcohol Exposure Paradigms.

A) Maternal drinking during pregnancy; B) Postnatal gavage/intragastric intubation; C) Postnatal exposure using a vapor chamber (dam with pups). Reproduced with permission from Boschen & Klintsova, *in press*.

2.3 Overview of Hippocampal Anatomy and Development

The hippocampus is still undergoing significant gliogenesis and synaptogenesis during the third trimester-equivalent, making it particularly vulnerable to alcohol exposure during this time window. To best understand how alcohol exposure impacts the hippocampus, knowledge of normal hippocampal anatomy and development is necessary. Figure 2.4 shows a representative image of the adult rat hippocampus (PD72).

It is estimated that the adult human hippocampus has 40-50 million neurons (Simic et al., 1997; West & Gunderson, 1990). Axons entering the hippocampus from lateral and medial entorhinal cortex (EC) via the perforant pathway make excitatory synapses onto the dendrites of granule cells in the dentate gyrus. Granule cells, which are the primary focus of Specific Aim 3, are found in the dentate gyrus and have small cell bodies which are tightly packed into a dense cell layer and have a cone-shaped apical dendrite extending into the molecular layer (Amaral et al., 2008). Granule cells have a single, thick, unmyelinated axonal projection (mossy fibers) which make primarily glutamatergic synapses onto CA2/3 pyramidal cells, other granule cells, and inhibitory hilar cells. Mossy fibers travel to CA3 through stratum lucidum and synapse within the pyramidal cell layer. One mossy fiber can contact one pyramidal cell multiple times and each pyramidal cell can receive input from up to 50 granule cells.

The pyramidal cells of CA3 have large cell bodies loosely distributed throughout the cell layer. Basilar dendrites extend into the stratum oriens and apical dendrites project into stratum radiatum. CA3 pyramidal cells have extensive reciprocal

connectivity with one another, and also send projections to the mammillary bodies via the fornix, the contralateral hippocampus via the hippocampal commissure, and pyramidal cells in CA1 via the Schaeffer collaterals which travel through the stratum radiatum.

The pyramidal cells in the CA1 are similar in structure to those in CA3 but are packed more densely into a cell layer. Apical dendrites reside in stratum radiatum and basilar dendrites extend into stratum oriens. The primary output for CA1 is the subiculum, though some axons are sent back to the EC. CA1 pyramidal neurons have been extensively studied for their role in spatial cognition as "place" cells. The primary output of CA1 and the subiculum is the thalamus, entorhinal cortex, and prefrontal cortex. Both CA3 and CA1 are also known to have reciprocal connections with the amygdala, particularly in ventral hippocampus.

The hippocampus is referred to as a "late-developing" structure, with development beginning around the 13th week of gestation and lasting at least until birth (Andersen et al., 2007). Most specific information known regarding the developmental timeline of the hippocampus is known through rodent studies, and as this developmental time course is more relevant to this dissertation, the following section will discuss the time course of morphological development based on what is known in these species. In rats, CA1 and CA3 pyramidal cells are generated first between embryonic days (E) 16-21, with the peak of CA3 pyramidal cell generation occurring slightly before CA1 cells on E17 compared to E18-19 (Bayer, 1980). Dentate gyrus granule cells begin to develop around E17, but continue to be generated

for up to three times as long, with only 15% of the granule cells actually being generated prior to birth in the rat. For all three regions, stem cells migrate from the ventricular germinative layer to the target region, though for granule cells, a secondary germinative matrix emerges in the hilar region which continues to populate the granule cell later during the postnatal period. Following birth, the rat hippocampus continues to undergo significant synaptogenesis, cell differentiation, neurite outgrowth, and changes to NMDA receptor conformation (Cline, 2001; Wenzel et al., 1997). Neurotrophic factors play a critical role in early hippocampal development; expression patterns of neurotrophins and their receptors change in a timing-dependent manner (discussed in more detail in Chapter 3) (Bernd, 2008).

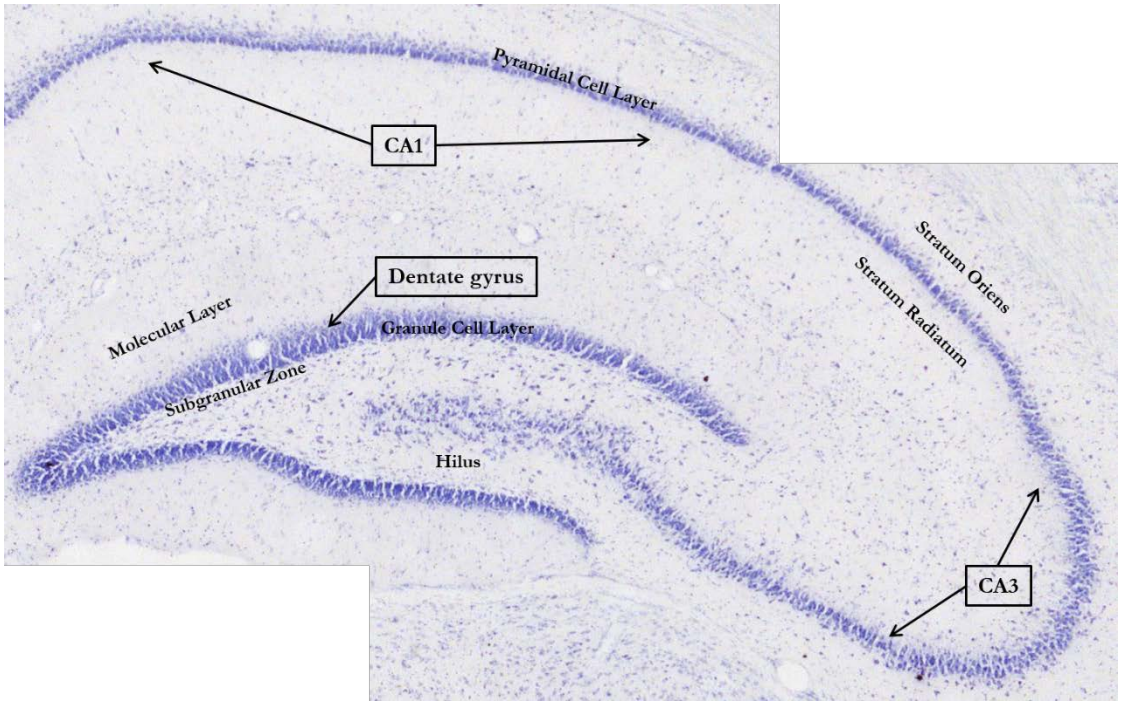

Figure 2.4. Representative Image of the Subfields of the Hippocampus.

Image taken with a 5x lens. Due to the limited field of view on the microscope, two images from the same section were stitched together. Section is stained with cresyl violet.

2.4 Developmental Alcohol Exposure and Hippocampal Dysfunction

Principal granule and pyramidal cell layer formation takes places during the second and third trimester-equivalents in rodents, making this time window of particular interest when studying the effects of developmental alcohol exposure on the hippocampus. This section will focus on the impact of developmental alcohol exposure on the structure and function of the hippocampus, though other regions will be discussed as well to illustrate the effect of alcohol on various processes when hippocampus-specific literature is limited. Additionally, while the hippocampus is the focus of this dissertation, this region is heavily interconnected with other areas that are

also targets of alcohol exposure; thus, the dysfunctions in certain up and downstream structures will be discussed

Alcohol is known to act as an NMDA receptor antagonist by reducing ion channel opening probability and open time. Though the precise nature of its antagonistic properties are still unclear, a direct interaction of alcohol with the NMDA receptor is likely due to the rapid reduction in channel activity (<100 msec) (Ron & Wang, 2009). NMDA receptors help regulate neuronal migration and differentiation in the developing brain, making disruption of NMDA receptor activity due to alcohol exposure all the more devastating during this time period (Komuro & Rakic, 1993). A short-term blockade of NMDA receptors in the late fetal or early neonatal period results in widespread apoptosis with a pattern of damage mimicking developmental alcohol exposure (Ikonomidou 1999; Thomas et al., 2001). NMDA receptors are also hypothesized to be particularly sensitive to damage through excitotoxic stimulation during the neonatal period (Ikonomidou et al., 1989), though it is not known if this hypersensitivity could extend to antagonism via alcohol exposure.

Alcohol acts a positive allosteric modulator of the $GABA_A$ receptor by increasing the passage of hyperpolarizing chloride into the cell. $GABA_A$ agonists, including alcohol, benzodiazepines, and barbiturates, induce apoptosis throughout the developing brain, compounding the cell death related to NMDA receptor antagonism following alcohol exposure. $GABA_A$ hyperactivation has the opposite effect in the adult brain, where activation of this receptor acts as a protective mechanism against NMDA-related excitotoxic apoptosis (Ikonomidou, 2000; Olney et al., 1991, 2001).

Thus, the fetus is far more sensitive to exposure to GABA mimetic agents compared to adults and alcohol presents a source of cell death on two fronts that work in concert. Normal neuronal maturation relies on a synchrony of signaling pathways, including GABA, glutamate and neurotrophic factors, to achieve proper cell differentiation, neurite outgrowth, and synaptic integration. GABA is particularly important during development prior to formation of glutamatergic synapses and has an excitatory effect in maturing cells; administration of alcohol to cultured neonatal hippocampal pyramidal cells disrupts GABA-mediated long-term potentiation (LTP) in these cells (Zucca & Valenzuela, 2010).

Rodent models of FASD have demonstrated that alcohol exposure affects LTP formation in the developing rat hippocampus, though this effect seems to be dose and timing-dependent. Administration of alcohol during the third trimester-equivalent disrupts LTP in neonatal CA1 *in vivo*, and *in vitro* models show that this effect is likely due to action on both the NMDA and AMPA receptor ion channels (Puglia & Valenzuela, 2010a,b). Age of the animal during LTP recording also likely makes a difference, as measurements of LTP in the adult rat brain following prenatal alcohol exposure tell a relatively different story, one that is highly dependent on sex of the animal. While male rats show reduced LTP in adulthood, female animals either exhibit no effect of alcohol exposure or an enhancement of LTP (Sickmann et al., 2014; Titterness & Christie, 2010). This observation in females seems to be independent of circulating estradiol (estrogen), as ovariectomized female rats present with the same pattern.

With the emergence of non-invasive technologies to study brain structure and function in humans, brain region volume, and cortical thickness became a few neuroanatomical measures well-characterized in children with prenatal alcohol exposure. Significant volumetric reductions have been reported in children with FASDs in regions such as the cerebellum, corpus callosum, caudate nucleus, parietal, and temporal lobes (Archibald et al., 2001; Autti-Ramo et al., 2002; ; Cortese et al., 2006; Fryer et al., 2012; Sowell et al., 2002). Changes to cortical thickness, white matter, and subcortical gray matter have also been found following prenatal alcohol exposure (Archibald et al., 2001), though the directionality of these alterations was region-specific, with greater cortical thickness observed in parietal and posterior temporal regions (Sowell et al., 2001, 2002, 2008; Fernández-Jaén et al., 2011; Yang et al., 2012) and decreased cortical thickness in frontal and occipital cortices (Zhou et al., 2011; Treit et al., 2014). However, increases in gray matter often corresponded with decreased white matter volume (Sowell et al., 2002) and white matter tracts in individuals with FASD are less organized compared to controls (Wozniak & Muetzel, 2011). The hippocampus also shows volumetric changes in individuals prenatally exposure to alcohol (Willoughby et al., 2008). Children diagnosed with FASD had smaller left hippocampi, poorer verbal learning, and impaired recall on both spatial and verbal tasks. Interestingly, in the FASD group, there was a positive correlation between performance on the recall tasks and hippocampal volume; no correlation was seen in the age-matched control group. Further, control children showed the expected

age-related increase in hippocampal volume, but volume did not increase in older children diagnosed with FASD.

Animal models of FASDs have also consistently demonstrated reductions in overall and region-specific brain volume (Bonthius & West, 1988; Napper & West, 1995; Maier et al., 1999; Sulik et al., 1984). Specifically, volume was decreased in regions including the cerebellum, brain stem, olfactory bulb, and hippocampus following postnatal alcohol exposure. High doses of alcohol from PD4-9/10 decreased the number of pyramidal cells in CA1 when assessed on PD10 and in adulthood (PD90), but CA2, CA3, and dentate gyrus were unaffected, suggested differences in regional susceptibility to damage (Bonthius & West, 1990, 1991). CA1 cell loss was also reported following exposure during a discrete neonatal period (PD7-9) (Marino et al., 2004). Developmental stage-dependence of hippocampal cell loss was demonstrated in two papers assessing the effect of binge-like alcohol exposure during the first, second, or third trimester-equivalents or all three combined (Livy et al., 2003; Tran & Kelly et al., 2003). CA1 cell loss was only found following that included the third trimester-equivalent, other exposure time points left the hippocampus unaffected. These two studies reported differing effects on dentate gyrus and CA3 cell numbers, with Livy and colleagues (2003) observing decreased cell counts and Tran and Kelly (2003) finding no changes. Similar inconsistencies have been reported in other literature, with some reporting CA3 and dentate gyrus cell loss (Miki et al., 2003, 2004) and others not (Bonthius & West et al., 1990, 1991), suggesting that differences

in dosing window, route of exposure, species, or age of tissue harvest could significantly influence findings.

Magnetic resonance microscopy and diffusion tensor imaging have also been useful tools in assessing structural changes following alcohol exposure. Using these techniques, high resolution images and detailed measurements of the fetal brain can be achieved. Alcohol exposure during the first and second trimester-equivalents results in larger lateral and third ventricles, a smaller pituitary and cerebellum, reduced cortical thickness and surface area, and agenesis of the corpus callosum and some septal regions (O'Leary-Moore et al., 2010; Leigland et al., 2013a,b; Parnell et al., 2013). E12-16 alcohol exposure results in reduced right hippocampal volume (Parnell et al., 2014); this timing of exposure would coincide with the very beginning of the formation of the CA1 and CA3 pyramidal cell layers. A further investigation of subregion-specific effects would be interesting, it is possible that the dentate gyrus would be less affected as the alcohol exposure ceases prior to its formation.

Alcohol-induced apoptosis at various developmental stages (Kerr et al., 1972; Creeley & Olney, 2013; Ikonomidou et al., 2000; Dunty et al., 2001; Climent et al., 2002; Olney et al., 2002) likely contributes to the long-term decreases in brain volume observed in both humans and animals exposed to alcohol prenatally. In the healthy brain, apoptosis is a naturally-occurring phenomenon critical to normal pre- and postnatal brain development (Johnston et al., 2009; Rakic & Zecevic, 2000). Prenatal or early postnatal alcohol exposure in rodents produces massive waves of apoptosis in the immature brain (Ikonomidou et al., 2000; Olney et al., 2002; Farber et al., 2010).

The hippocampus has been shown to be highly vulnerable to alcohol-related cell loss, particularly during the neonatal period (Ikonomidou et al., 2000; Heaton et al., 2003; Livy et al., 2003; Wozniak et al., 2004). Perinatal alcohol exposure (35 days total) increased the number of TUNEL+ cells (apoptotic marker) in all subregions of the hippocampus (Shirpoor et al., 2009). However, even acute exposures to alcohol during the early postnatal period can induce apoptosis. A one or two day binge of alcohol during the third trimester-equivalent increased rates of apoptosis in the hippocampus (Smith et al., 2015). Alcohol-induced apoptosis is likely driven by a silencing of neuronal and network activity following the suppression of glutamatergic NMDA receptor signaling and potentiation of GABAergic receptors (Lebedeva et al., 2015). Lack of appropriate and sufficient synaptic input will cause activity-dependent apoptosis.

Another route through which alcohol can stimulate apoptosis in the hippocampus and other brain regions is through induction of oxidative stress pathways. Oxidative stress damages cells and DNA through the production of peroxidases and other free radical molecules, leading to increased cell death. Developmental alcohol exposure has been repeatedly demonstrated to increase markers of oxidative stress in the neonatal and adult rodent brain. Prenatal exposure increased levels of reactive oxygen species (ROS) and apoptosis in fetal rat cortical neurons, while antioxidant pretreatment of the cells prevented the apoptosis, meaning ROS production directly contributed to increased alcohol-induced cell death (Ramachandran et al., 2003). Lipid peroxidase was also upregulated throughout the

brain for up to 12 weeks following postnatal alcohol exposure (Petkov et al., 1992) and until at least PD60 following perinatal exposure (Brocardo et al., 2012). Perinatal alcohol also increased protein oxidation in the hippocampus and cerebellum and increased anxiety- and depressive-like behaviors in the mice.

Hypoperfusion of blood to target organs during embryonic development has been hypothesized to contribute to low birth weight and the cognitive impairments observed in children with FAS. This hypothesis has been supported by studies showing that children with FAS have lower cerebral blood flow, with considerable hypoperfusion of the temporal lobe specifically (Riikonen et al., 1999; Bhatara et al., 2002). Similar findings have been borne out using animal models. Alcohol administration in adult rats decreased vasodilatory response to a variety of drugs that increase microvessel diameter in the healthy brain (Mayhan et al., 1992). *In utero*, alcohol-exposed fetuses experience reduced placental and arterial blood flow to the brain (Jones et al., 1981; Bake et al., 2012). Inspection of brain sections taken from prenatally exposed mice revealed a disorganized and dying microvasculature (Jégou et al., 2012).

Altered blood flow and microvessel density following alcohol exposure could affect the hippocampus in a unique way. The dentate gyrus continues to generate new cells throughout the lifespan, a process known as adult neurogenesis. The successful proliferation of cells in the subgranular zone of the dentate gyrus relies on the establishment of a neurogenic niche, areas rich in nutrients, oxygen, and growth factors which support the local stem cell population (Doestch, 2003). Neuronal

precursors in the subgranular zone eventually migrate into the granule cell layer and mature into fully functional granule cells. Compromised blood flow into the stem cell niche could limit the number of progenitor cells able to successfully divide and impact the survival of these adult-born neurons. Rodents models of FASD have demonstrated impaired adult neurogenesis (Redila et al., 2006; Ieraci et al., 2007; Klintsova et al., 2007; Gil-Mohapel et al., 2011; Hamilton et al., 2011; Ieraci et al., 2007; Singh et al., 2009), possibly contributing to the plethora of hippocampal-associated behavioral deficits observed in these paradigms. The impact of developmental alcohol exposure on the stages of adult neurogenesis will be discussed in more detail in Chapter 4 (Foundational Studies).

2.5 Developmental Alcohol Exposure Alters Learning, Anxiety- and Depression-like Behaviors

Children with FASDs exhibit both scholastic and intellectual disabilities (Mattson et al., 1996a, b; Mattson et al., 1999; Rasmussen et al., 2011; Mattson et al., 1997), as well as abnormal social and emotional interactions. As adults, individuals with FASDs are more likely to have trouble with the law, in part due to executive functioning and impulse control deficits (Irner et al., 2012; Stevens et al., 2012; Franklin et al., 2008; Kodituwakku et al., 1995; Pei et al., 2011; Streissguth et al, 2004). To some degree, the learning and memory dysfunctions observed in humans with FASDs (Uecker & Nadel, 1996) have been modeled in animals as well. In rodent models, alcohol-induced alterations to behavioral endpoints is largely dependent on the timing and dose of the exposure, however both prenatal and postnatal models can

significantly disrupt hippocampal-associated behavior (Berman & Hannigan, 2000; Thomas et al., 1996, 1997; O'Leary-Moore et al., 2006; Hunt et al., 2009; Hamilton et al., 2012). These deficits include impairment on spatial alternation tasks, the Morris Water Maze, the radial arm maze, discrimination and reversal learning, and variants of fear conditioning. Specifically, rats exposed to alcohol from PD4-9 show deficits on trace, but not delay, fear conditioning (Wagner & Hunt, 2006). Trace fear conditioning, which incorporates a longer delay ("trace interval") between the presentation of the conditioned stimulus (e.g. a tone or light) and the shock, relies more heavily on intact hippocampal function compared to delay fear conditioning (McEchron et al., 1998). Third trimester-equivalent alcohol exposure (PD4-9) also impairs the ability of rats to learn a novel context during the hippocampus-dependent context pre-exposure facilitation effect variant of context fear learning and reduces novelty-associated c-Fos expression in hippocampal CA1 (Murawski et al., 2012). Performance on another commonly used hippocampal-associated spatial task, the Morris Water Maze (MWM), is also impaired in rodent models of FASD, though these deficits are dependent on timing of exposure and blood alcohol concentration (Goodlett & Johnson, 1997). During the MWM task, rodents are placed in a large pool of opaque water and must locate a submerged platform based on spatial cues. There are two variants of this task, one in which the rat is placed into the pool in the same spot every time and can learn to find the platform by always following the same path (hippocampus-independent) and one in which the starting point is changed for each trial, causing the rodent to rely on spatial awareness to find the platform

(hippocampus-associated). High, binge-like doses of alcohol during specific time windows (PD7-9 or PD4-9 seem to be most consistent) cause deficits in maze acquisition on the hippocampus-associated version of the task (Kim et al., 1997; Johnson & Goodlett, 2002; Marino et al., 2004).

Beyond performance on spatial memory tasks, alcohol-exposed animals also show alterations to social and anxiety behaviors. Third trimester-equivalent alcohol exposure (PD4-9) reduced exploratory and play behaviors in adolescent rats housed in a complex environment with enhanced social and novelty-related stimulation (Boschen et al., 2014), and alcohol-exposed rats display altered play fighting and decreased social recognition behaviors in other social interaction paradigms (Kelly & Dillingham, 1994; Kelly et al., 2009; Middleton et al., 2012; Mooney & Varlinskaya, 2011). Children with FAS also show social behavior deficits which present independent of changes to IQ (Thomas et al., 1998). There is some evidence that alcohol-exposed animals show anxiety- and depressive-like behaviors, such as increased learned helplessness and immobility during the forced swim test, which were correlated with downregulation of *Bdnf* expression in the medial prefrontal cortex (mPFC) and hippocampus (Hellemans et al., 2008; Caldwell et al., 2008). Depression is more commonly diagnosed in children with documented prenatal alcohol exposure compared to age-matched controls (Roebuck et al., 1999). Increased presentation of psychopathology is apparent from early childhood and the risk continues through adolescence, at the least (O'Connor et al., 2000, 2006; Steinhausen & Spohr, 1998). Like social functioning deficits, anxiety and depression are associated

with prenatal alcohol exposure even if the children had a normal IQ and performance other measures of intelligence. Whether the increased propensity of children with prenatal alcohol exposure to develop depression and anxiety is related to altered neurotrophin functioning within the prefrontal cortex or limbic areas remains to be investigated.

2.6 Conclusions

Alcohol exposure during brain development has a devastating impact on hippocampal structure and function through a variety of mechanisms. These deficits are apparent both in children diagnosed with FASD and in animal models. The type and severity of hippocampal damage depends largely on factors including the dosing window and peak blood alcohol concentration. Ultimately, these neuroanatomical and functional impairments result in cognitive and behavioral deficits on tasks associated with normal hippocampal function. The hippocampus is a highly plastic structure throughout the lifespan and is sensitive to both positive and negative environmental stimulation. Understanding the role of neurotrophic factors in the hippocampus and how behavioral interventions which enhance neuroplasticity affect hippocampal structure and function is necessary for developing therapies to ameliorate the neuroanatomical and behavioral deficits I outlined in this chapter. Chapter 3 will discuss the importance of neurotrophins in development and neuroplasticity throughout life, with a focus on brain-derived neurotrophic factor (BDNF) and the influence of exercise and environmental complexity on the adult hippocampus. Chapter 4 will tie these themes together to discuss how neonatal alcohol exposure

impacts measures of neuroplasticity, including adult neurogenesis, dendritic complexity, and BDNF levels, as well as our laboratory's work demonstrating wheel running and housing in a complex environment as promising therapeutic avenues for alcohol-related deficits.

Chapter 3

NEUROTROPHINS, NEUROIMMUNE FUNCTION, AND ENVIRONMENTAL MANIPULATIONS: IMPACT ON HIPPOCAMPAL DEVELOPMENT AND NEUROPLASTICITY

The goal of this chapter is to discuss three factors which may interact with developmental alcohol exposure to either exacerbate or protect against alcohol-induced damage: neurotrophic factors, the neuroimmune response, and environmental manipulations, specifically aerobic exercise and housing in a complex environment. Neurotrophic factors and the neuroimmune system are critical for proper brain development and alterations to these normal developmental processes can impact brain structure and function across the lifespan. These sections (3.1 and 3.2) will discuss the current literature in regards to the role of neurotrophic factors (focusing on the neurotrophin BDNF) and the neuroimmune response in brain development, neuroplasticity, and behavioral output. As with Chapter 2, the primary focus will be the hippocampus, but other brain regions will be discussed as well. Section 3.3 will discuss exercise and environmental complexity, the two behavioral therapies used in our laboratory to rescue alcohol-associated deficits in neuroanatomy and behavior, in regards to their impact on hippocampal neuroplasticity and neurotrophin levels[2].

[2] Text in this chapter has been reproduced or adapted with permission from Boschen & Klintsova, *in press*.

3.1 Role of Neurotrophic Factors in Brain Development and Neuroplasticity

Neurotrophic factors include three families of growth-related molecules critical for a cell proliferation, synaptogenesis, cellular structure, and maintenance of microvessels throughout the brain. One family, neurotrophins, play a key role in proper brain development and are important mediators of synaptic plasticity involved in memory formation during adulthood. Neurotrophins include neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF). This chapter will primarily discuss the neurotrophins, with focus being BDNF as it is the neurotrophin analyzed in the experimental portion of this dissertation; however, the neurotrophic factor vascular endothelial growth factor (VEGF) will also be discussed due to our emerging understanding of its importance in neuroplasticity, including establishment of the neurogenic niche and adult neurogenesis. Due to their importance both during early development and in the adult brain, disruption of neurotrophins or receptor function during the prenatal or early postnatal period could result in long-term changes to cognition, memory formation, and mood.

3.1.1 Expression of Neurotrophic Factors During Development

The proper timing of the birth, survival and death of neurons is carefully orchestrated with the help of neurotrophin signaling. Each neurotrophin exhibits a unique expression profile throughout development in the brain and periphery, with alterations to expression causing significant and long-term consequences on cell structure and function. Studies in chicken embryos show NT-3 as the earliest

neurotrophin found in the developing embryo, with expression of its receptor, tropomyosin receptor kinase C (TrkC), beginning in the neural plate (Bernd, 2008). BDNF and its receptor, TrkB, emerge later, appearing first in the neural tube. NGF and its receptor, TrkA, is first expressed in peripheral sensory neurons later in embryonic development, suggesting a role for this neurotrophin in later developmental stages rather than initial cell proliferation and migration processes. Another neurotrophic factor, VEGF, is critical for embryonic vasculogenesis. VEGF-A, VEGF-B, VEGF-C, and VEGF-D have three receptors, VEGFR1-3, with VEGF-A and its receptor VEGFR2 being the most plentiful in the brain. Exposure to toxins or stress during gastrulation and neurulation could influence expression of these neurotrophins in a timing-dependent manner.

Neurotrophins also show overlapping but distinctive patterns of expression during later brain development. In rat embryos, levels of NT-3, NGF and BDNF increase to coincide with mass neurogenesis in the developing central and peripheral nervous system around embryonic day (E) 11-12 (Maisonpierre, 1990). During these first waves of neurogenesis, NT-3 is the most abundantly produced neurotrophin, with BDNF being far less plentiful, suggesting a prominent role of NT-3 during initial neurogenesis. In addition to being developmentally regulated, neurotrophic expression is also brain region-specific, with expression of NT-3, NGF and BDNF decreasing in concentration in the adult vs. prenatal spinal cord; conversely, expression of NGF and BDNF increase into adulthood in the hippocampus (Timmusk et al., 1994; Katoh-Semba et al., 1998; Karege et al., 2002) while expression of NT-3 peaks in the early

postnatal period (equivalent to third trimester of human pregnancy). Based on these expression profiles, NT-3 might play a more important role in early neuronal proliferation and migration, with NGF and BDNF contributing to later developmental processes, such as neuronal survival, neurite outgrowth and synaptic plasticity.

3.1.2 Neurotrophins Influence Hippocampal Structure and Function

Neurotrophins are highly expressed in the adult rodent hippocampus and act through a variety of pathways to influence pro-neuroplastic gene transcription in this region, including the ERK1/2-CREB and PI3-K-Akt pathways. Additionally, BDNF can activate phospholipase C-γ and CAMK-II pathway, resulting in increased insertion of AMPA receptors into the postsynaptic membrane. Neuronal activity reciprocally promotes further neurotrophin gene transcription via CREB. For instance, while neurotrophins do not act directly on glutamate receptors, NMDA- and non-NMDA-mediated glutamatergic activity can enhance synthesis of BDNF and NGF in the rodent hippocampus (Zafra, 1990; Lindholm et al., 1994). The interaction between NMDA receptors and neurotrophins is important as increased activity in a neural system could induce neurotrophic factor synthesis and release to supplement other plasticity-related processes. Activity-dependent BDNF release caused through NMDA receptors can regulate anti-apoptotic signaling cascades through the TrkB receptor; blockade of this receptor disrupts the anti-apoptotic effect (Bhave et al., 1999). Neurotrophic factors, namely BDNF, have also been shown to be necessary for protein synthesis-dependent, late-phase LTP in the neonatal and adult rodent brain. Even in the presence of protein synthesis inhibitors, administration of BDNF can maintain

LTP *in vitro* (Lu & Chow, 2007) and inhibition of BDNF through antagonists or BDNF scavengers can eliminate previously stable LTP. In the neonatal rat, high frequency stimulation often only produces short-term potentiation due to synaptic fatigue; administration of BDNF allows for induction of LTP in the neonatal brain (Gottschalk et al., 1998). Some authors have posited that it is no coincidence that levels of BDNF increase in the neonatal brain alongside the ability to maintain LTP (Figurov et al., 1996). One way in which BDNF is thought to enhance maintenance of LTP is through increased vesicle docking and neurotransmitter release from the presynaptic terminal (as opposed to direction interaction with AMPA or NMDA receptors). Mutant BDNF knockout mice display fewer docked vesicles and decreased levels of vesicle docking proteins synaptophysin and synaptobrevin (Pozzo-Miller et al., 1999); treatment with BDNF attenuates these effects. Increases in vesicle docking due to BDNF have functional significance, resulting in increased frequency of AMPA-mediated spikes which are connected to vesicle, and thus neurotransmitter, release (Tyler & Pozzo-Miller, 2001). Aside from BDNF, there is evidence that VEGF may play a role in LTP and memory formation, as overexpression of VEGF *in vivo* enhanced LTP but blockade of VEGF reduced LTP to normal levels (Licht et al., 2011). These data suggest importance of VEGF for enhancement of LTP but not necessarily induction and maintenance of "normal" LTP. The mechanisms through which VEGF affects LTP remain to be determined.

BDNF and other neurotrophins have shown promise for their role in neuroprotection (Pezet & Malcangio, 2004; Davis, 2008; Idrus & Thomas, 2011),

preventing apoptosis following excitotoxicity and ischemic hypoxia (Almeida et al., 2005; Han et al., 2000). One pathway through which BDNF and other neurotrophins might offer neuroprotection against apoptosis is through regulation of oxidative stress pathways. As discussed in Chapter 2, these pathways are a likely target of alcohol exposure and contribute to alcohol-induced apoptosis. Neurotrophins have been shown to combat oxidative damage in various disease models through upregulation of antioxidant molecules. Alternatively, ROS inhibit neurotrophins (Gardiner et al., 2009). Diets high in saturated fat increase oxidative stress-related damage and reduce BDNF. Vitamin E supplementation decreased tissue damage and upregulated BDNF, possibly through phosphorylation of synapsin I and activation of CREB signaling pathways (Wu et al., 2004). NGF has also been shown to reduce oxidative damage to synaptic transporters by β-amyloid and iron molecules (Guo & Mattson, 2000). Cultured hybrid cells made from mitochondrial DNA of Parkinson's disease patients had enhanced vulnerability to oxidative damage and reduce glutathione presence; however administration of either BDNF or glial cell line-derived neurotrophic factor protected the cells from oxidative stress-induced death (Onyango et al., 2005). Finally, most relevant to the current topic, BDNF, NGF and NT-5 all showed neuroprotective effects on oxidative damage in the neonatal brain (Kirschner et al., 1996). Administration of 1-methyl-4-phenylpyridinium (MPP+) into the PD8 striatum caused significant hypoxic lesions to the tissue which were reduced in volume by systemic administration of neurotrophic factors prior to MPP+ injection. While the exact mechanisms of the neurotrophin-oxidative stress interaction are still unclear, it has

been proposed that a positive feedback loop between neurotrophin and antioxidant production exists in the healthy brain (Gardiner et al., 2009).

Cerebral blood flow and microvasculature are compromised follow developmental alcohol exposure and represent another area where neurotrophins could have a compensatory function. Establishment and maintenance of the blood-brain barrier is essential to normal brain development, function, and plasticity. Neurotrophins enhance microvasculature and promote blood-brain barrier permeability. Treatment with BDNF prior to a spinal cord injury can even offer limited protection against injury through opening of the blood-spinal cord barrier (Sharma, 2003). BDNF is thought to have dual roles in enhancement of angiogenesis: promotion of endothelial cell survival through action at the TrkB receptor and through recruitment of immune cells to the site of an injury (Kermani & Hempstead, 2007). BDNF is an important part of ischemic injury repair in the skin, arterial and cardiac tissues. NGF has been show to support angiogenesis in certain preparations as well (Cantarella et al., 2002). Perhaps the most important neurotrophin for the microcirculatory system and angiogenesis is VEGF, particularly vasculogenesis during development. In addition, VEGF is critical in the formation and restructuring of blood vessels following injury and establishment of the neurogenic niche in the hippocampus (Ferrara et al., 2003; Rosenstein & Krum, 2004; Alvarez-Buylla & Lim, 2004). VEGF has been tested as a protective agent in hypoxic or ischemic injury (Ellis & Hicklin, 2008), though the success of these trials has been limited, not a least in part due to the

complex timing of events during hypoxic injuries and mechanistic underpinnings of VEGF signaling that remain to be addressed.

Proper neurotrophin signaling is important for all stages of adult neurogenesis, though certain neurotrophins seems to be more critical during certain cell maturation processes (Maisonpierre et al., 1990). Based on work showing that TrkB receptor expression is low during the first week following cell birth and increased as the cell ages, BDNF is likely more important for later cell maturation, not initial proliferation (Donovan, 2008). Sairanen and colleagues (2005) also reported that both BDNF and TrkB mutant mice displayed disrupted cell survival 21 days following injection of bromodeoxyuridine (BrdU) which labels progenitors actively going through the S-phase of the cell cycle. These mice also failed to show increased cell survival following administration of a selective serotonin reuptake inhibitor (SSRI) which increased cell survival in wild-type mice, suggesting that the SSRI's effect was through interaction with BDNF. Chan and colleagues (2008) published an elegant study investigating granule cell maturation in conditional BDNF mutant mice which had ~50% of wild-type levels of BDNF in the adult hippocampus. These mice demonstrate stalled neuronal maturation and disrupted migration of adult-born cells. Specifically, the newly generated granule cells failed to migrate as far into the granule cell layer. Recent work has begun to establish a role for VEGF in adult neurogenesis, both indirectly through establishment of a neurogenic niche and by directly enhancing cell maturation processes (Licht et al., 2011).

Neurotrophins play an important and well-orchestrated role in dendritic morphology and spine density throughout the brain. In the cortex, BDNF and NT-3 have been shown to oppose one another's impact on dendritic structure depending on the cortical layer (McAllister et al., 1997). In Layer IV, BDNF enhanced dendritic growth while NT-3 administration blocked this effect. In Layer VI, the roles were reversed, with BDNF inhibiting and NT-3 administration stimulating dendritic growth. BDNF has been repeatedly shown to enhance dendritic length and complexity in the hippocampus and in cell culture (Singh et al., 2006; Tolwani et al., 2002; Alonso et al., 2002). Interestingly, in some preparations, the effect of BDNF was quite local and only affected apical vs. basilar CA1 dendrites (Alonso et al., 2002). Additionally, in this paper, the effect of BDNF was limited to dendritic length, with number of bifurcations unaltered following treatment. Conditional knockdown of BDNF in the mouse hippocampus causes granule cells to develop simpler and shorter dendritic arborizations (Chan et al., 2008) and transfection of BDNF into dentate gyrus cell cultures enhanced dendritic outgrowth, even causing the growth of basilar dendrites on some granule cells which do not occur naturally (Danzer et al., 2002). BDNF also increases spine density on CA1 apical dendrites through the ERK1/2 pathway (Alonso et al., 2002; Tyler & Pozzo-Miller, 2003). Aside from increasing spinogenesis, BDNF administration can cause spines to take on a more unstable, filopodia-like phenotype (Shimada et al., 2002; Horch et al., 1999), possibly priming the dendrite for upcoming changes to intracellular signaling. The enhancing effects of BDNF are likely through action at the TrkB receptor, as activation of the p75 receptor seems to have the

opposite effect of BDNF administration by decreasing dendritic complexity and spine density (Zagrebelsky et al., 2005).

3.1.3 Neurotrophins Enhance Learning and Reduce Anxiety- and Depressive-like Behaviors

Through their actions on the molecular and anatomical systems of the brain, neurotrophin levels are correlated with a wide range of behavioral phenotypes. BDNF release in the hippocampus has been shown in multiple animal models to be required for short- and long-term contextual and spatial memory formation (Lu & Chow, 1999; Alonso et al., 2002; Griffin et al., 2009). BDNF is rapidly upregulated during contextual memory formation (Hall et al., 2000) and blockade of hippocampal BDNF release impairs the ability of mice to form hippocampus-dependent memories (Heldt et al. 2007). Exercise, which robustly enhances neurotrophin production in the hippocampus and perirhinal cortex, also improves performance on both spatial and non-spatial memory tasks (Griffin et al., 2009). Supporting the role of BDNF in the behavioral enhancements, injection of BDNF directly into the cerebroventricular system resulted in the same augmentation to memory. Mechanistically, BDNF increases levels of docked synaptic vesicles and neurotransmitter release, leading to enhanced LTP induction and maintenance, making BDNF a critical variable in the synaptic and cellular changes underlying memory formation.

Neurotrophin dysregulation has also been implicated in anxiety and depressive-like behaviors in rodent models of mood disorders (Martinowich et al., 2007). Some studies have found that decreases in BDNF have been correlated with

behavioral phenotypes of depression and anxiety, but often these effects are small and inconsistent between models and differ between male and female animals. More convincing is the upregulation of BDNF following antidepressant treatment with selective serotonin reuptake inhibitors (SSRIs), and evidence suggests that regulation of BDNF expression and signaling is a downstream target of these drugs. *Bdnf* mutant mice which express increased anxiety-like behaviors did not show improvements following treatment with SSRIs, suggesting BDNF upregulation as a key component of antidepressant action (Chen et al., 2006). In humans, the Val66Met polymorphism has been linked to reduced hippocampal volume and the development of psychopathologies including depression, anxiety, and schizophrenia (Sanchez et al., 2011; Martinowich et al., 2007). This single-nucleotide polymorphism refers to a methionine (Met) substitution for valine (Val) in the pro-domain (codon 66) of the BDNF protein and disrupts activity-dependent release of BDNF (Chen et al., 2005). While the Val66Met polymorphism increases an individual's genetic risk for developing psychopathology, experiencing early life stress or trauma can enhance this risk (Gatt et al., 2009). Mouse models of this polymorphism ($BDNF^{Met/Met}$) also show increased anxiety-related behaviors, including reductions in time spent in the center of an open field and in the open arms of an elevated plus maze (Chen et al., 2006). These behavioral changes were not reversed with administration of an SSRI and were accompanied by decreased hippocampal volume and dendritic complexity. Depression- and anxiety-like symptoms are linked with medial temporal lobe dysfunction, suggesting that disruption of neurotrophin signaling within the

hippocampus and connected structures could play a role in the development of psychopathologies.

3.2 Role of the Neuroimmune System on Brain Development and Neuroplasticity

Microglia are the resident immune cells of the brain. They respond to injury, infection, or exposure to toxins or drugs (such as alcohol) by releasing pro- and anti-inflammatory cytokines and chemokines and phagocytosis of dying cells and debris (Fu et al ., 2014). In the healthy brain, microglia play a critical role in synapse maintenance, neurogenesis, apoptosis, and even learning and memory. Dysregulation of the developing immune system has the capacity to shape long-term microglial morphological and chemical activation patterns to immune challenges, possibly contributing to the emergence of neurological and psychological disorders in adulthood, including schizophrenia and depression (Schwarz & Bilbo, 2011, 2012). This dissertation seeks to investigate alcohol-induced microglial activation as both a secondary source of damage and as a potential avenue for therapeutic intervention for children prenatally exposed to alcohol. This section will describe developmental events related to microglial colonization of the brain, the function of microglia and cytokine expression in the developing CNS, and the consequences of aberrant early neuroimmune activation on cognition and behavior in adulthood. Additionally, I will discuss the interaction of neurotrophins and the neuroimmune system.

3.2.1 Microglial Colonization, Morphology, and Cytokine Expression in the Developing CNS

Macrophage precursors originating from the embryonic yolk sac begin to colonize the developing brain by the 5th or 6th week of gestation in humans and E9-10 in rodents (Chan et al., 2007; Ginhoux et al., 2010; Schwarz & Bilbo, 2011, 2012). Microglia are apparent by week 7-8 of gestation in humans (E13-14 in rodents). The first microglia have an activated, amoeboid phenotype but develop short, thick processes and transform to a ramified morphology through the remainder of fetal and early postnatal development, with phenotypically mature microglia comprising the majority of these immune cells by PD14 in rodents (Bilbo & Schwarz, 2009). Colonization takes place in a region-specific manner, with microglial appearing in the spinal cord prior to cortical regions. Hippocampal colonization takes place rather early in this time line, likely due to its proximity to the corpus callosum and ventricles. During the early postnatal period, corresponding with the third trimester-equivalent, most microglia in the rodent hippocampus have distinct processes, ranging from short and thick to longer, thinner processes resembling the mature ramified phenotype. Ramified microglia are often referred to as “quiescent” or “resting” but this terminology could be considered a misnomer due to their dynamic and motile state (Nimmerjahn et al., 2005).

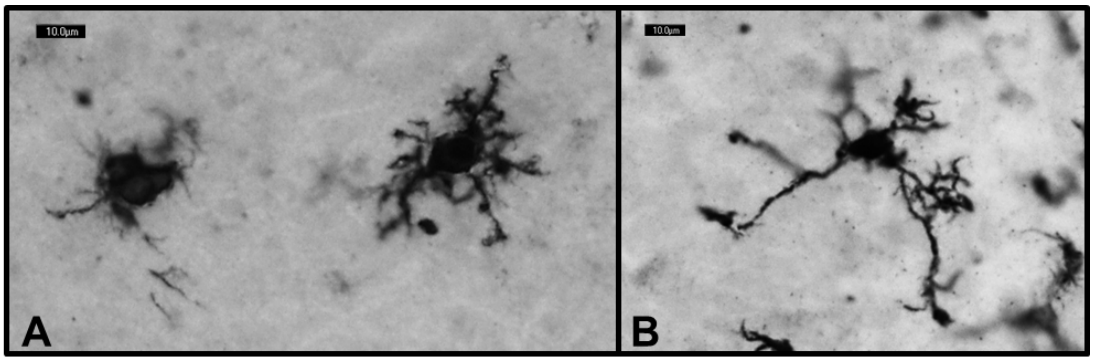

Figure 3.1. Representative Images of Microglia in the PD10 Male Rat Hippocampus.

A) These microglia are indicative of activated microglia. The microglia on the left has a very large, round soma with very few, short processes. To the right, this microglia is less activated. The soma is slightly smaller and the microglia has more processes compared to the one on the left, however, these branches are still short and thick. B) This microglia has a ramified phenotype with longer, thin processes and a small cell body. Both images taken with a 40x lens, scale bars = 10 µm.

Cytokine expression is upregulated in the developing brain, possibly as a means to attract infiltrating microglial precursors (Schwarz & Bilbo, 2011, 2012). Members of the C-C motif chemokine ligand family, such as CCL2 and CCL4, are highly expressed at PD0 in the rat hippocampus and cortex, though these levels drop dramatically across the first four postnatal days (Schwarz et al., 2012a, b). These cytokines are not necessarily expressed by the microglia themselves, instead being expressed by stem cells and glia cells. This distinction is not only important for their possible role in the developing brain, as a chemical attractant to infiltrating microglial precursors, but also important for the interpretation of infection or toxin-induced upregulation of these cytokines.

The interleukin (IL) family members, which include the well-studied cytokines IL-1, IL-6, and IL-10, have differential patterns of expression throughout brain development and can pass the placental barrier from mother to fetus (Dahlgren et al., 2006). IL-1 appears following the appearance of amoeboid microglia in the rat brain, with cerebral protein levels peaking around E18-20 (Giulian et al., 1988). The number of amoeboid microglia in the cerebrum also peaks at this age, suggesting these microglia as the source of the IL-1. Support for this claim was found following administration of L-leucine methyl ester to amoeboid microglial cultures. This agent lyses and destroys this specific phenotype of microglia through disruption of the cell's lysosomes and induction of apoptosis following internalization of the compound by the microglia (Jebelli et al., 2015). Depletion of the amoeboid microglia resulted in a corresponding reduction in levels of IL-1 (Giulian et al., 1988). In contrast, levels of IL-6, are relatively low at birth but increase dramatically by PD20 in the rat hippocampus, striatum, and cortex (Gadient & Otten, 1994; Schwarz et al., 2012). IL-6 seems to influence cell differentiation, though this effect could be through its regulation of some neurotrophic factors (Frei et al., 1989). As the levels of IL-6 did not increase as significantly in all structures (e.g. cerebellum, brain stem), suggesting a region-specific function for cytokine in postnatal development. IL-10 is an anti-inflammatory cytokine which exists at very low levels in the healthy brain and is upregulated in response to trauma or infection. In fact, one study in human neonates found significantly lower levels of IL-10 in the healthy infants compared to adults; moreover, when blood cell cultures taken from the infants were stimulated to induce

an immune response, the upregulation of IL-10 was less profound in the neonates (Schultz et al., 2004). Even more interesting, supplementation of recombinant IL-10 in the cultures did not inhibit pro-inflammatory cytokine production to the same degree seen in adults. These findings suggest that the anti-inflammatory response is not mature in young infants. IL-10 has been found to be upregulated in blood following sepsis in infants (Ng et al., 2003), suggesting that a severe infection is needed to stimulate production.

Other cytokines of interest for this dissertation include cluster of differentiation molecule 11b (CD11b; also known as integrin alpha M or ITGAM), tumor necrosis factor-α (TNF-α), and the anti-inflammatory cytokine transforming growth factor-β (TGF-β). CD11b levels increase in the developing (PD0) hippocampus and cortex as the animal ages, with upregulation apparent by PD4 (Schwarz et al., 2012). TNF-α is detected as early as E30 (first trimester-equivalent) in the sheep, though levels remain low and almost disappear by birth (Dziegielewska et al., 2000). In one study, levels of TNF-α protein were similar to IL-6 levels in the E16 rat brain, suggesting relatively low expression (Urakabo et al., 2001). Expression of both of these cytokines was lower than those reported for IL-1β at this time point (~1500 pg/g brain tissue for TNF-α compared to ~5000 pg/g for IL-1β). Interestingly, both TNF-α and IL-6 were expressed in the amniotic fluid whereas levels of IL-1β were undetectable. TGF-β has three isoforms, TGF-β1-3, which have multiple functions within the brain and the peripheral nervous system. All three isoforms were detected in the embryonic meninges, and TGF-β2 and 3 were detected in glia and the choroid plexus from E12.5-

18.5 (Pelton et al., 1991). These findings suggest a role for TGF-β in development of the blood-brain barrier. Another study reported low levels of TGF-β2 in the brain around E11 in the mouse, however, in general the levels were too low to quantify (Millan et al., 1991). It is important to note that significant sex differences exist regarding the number of microglia and cytokine expression in the developing brain (Schwarz et al., 2012). As this dissertation focused on male rat pups, the discussion will be framed in that regard.

3.2.2 Neuroimmune Activation during Development has Long-term Consequences on Brain Function

Precise regulation of microglial function is necessary for proper neuronal development and a long-term normal cognitive and behavioral trajectory. A multitude of studies have linked early life programming of the immune system by infection to later development of diseases, mood disorders, and neuropsychiatric conditions, neuropsychiatric disorders such as depression, post-traumatic stress disorder (PTSD), and schizophrenia (reviewed in Watanabe et al., 2010, Schwarz & Bilbo, 2012; Dantzer, 2009). In fact, it is well-accepted that maternal infection during gestation is a significant risk factor for the development of schizophrenia (Müller & Schwarz, 2010). These disorders can be modeled in rodents using tasks to assess anxiety (acoustic startle, elevated plus maze) and memory for a fearful stimulus or context.

Various models of early immune activation exist, but the most commonly used are systemic injection of lipopolysaccharide (LPS), molecules which comprise part of the cell wall of gram-negative bacteria, or the bacteria *E. coli*. While LPS does not

pass the blood-brain barrier, it can still induce a microglial response and upregulation of cytokine production. Experiments by Walker and colleagues (2004; 2006; 2008) demonstrated that administration of LPS to neonatal pups (PD3 and 5) causes increased levels of corticosterone 4 hours post-injection and in adulthood, an exaggerated acoustic startle and corticosterone response following stress, and increased anxiety behavior on the elevated plus maze in adulthood and elderliness. Further studies by Bilbo and colleagues (2005; 2008; 2010) showed that PD4 infection with *E. coli* showed impaired contextual memory, reduced expression of BDNF in the hippocampus following contextual fear conditioning, and disrupted spatial memory (only at 16 months of age). Interestingly, many of the behavioral alterations were linked to the presence of a second immune challenge following training. This observation supported what is known as the "two-hit" model, meaning that an immune challenge during development and then again during adulthood could lead to, and are in some cases necessary for, development of neuropsychiatric disorders (Dantzer, 2009; Schwarz & Bilbo, 2012). Furthermore, early life activation of the immune system has primed the brain and body to respond to a second immune challenge with hyperactivation of microglia and hypersecretion of cytokines (Bilbo & Schwarz, 2009).

While the exact mechanisms of how immune activation during development primes the brain and body for later dysfunction, it is likely that cytokine induction plays an important role. For example, the review by Watanbe and colleagues (2010) discusses a series of studies from their group showing that subcutaneous injection of

cytokines (including IL-1α, TNF-α, and IL-6) to neonatal pups from PD2-10 altered performance on startle and pre-pulse inhibition tasks in adolescence and adulthood. Certain cytokines also contribute to normal neuronal processing in the healthy brain, meaning that dysregulation of these cytokines during illness could directly impact cognition (McAfoose & Baune, 2009). In recent years, our understanding of the importance of IL-1 in normal synaptic function and learning and memory processes has grown considerably. In the adult brain, IL-1 is expressed by both glia and neurons and is heavily concentrated in the hippocampus (Schneider et al., 1998). Beyond upregulation in response to infection or injury, IL-1 is an important molecule for learning and memory processes. IL-1 is upregulated following induction of LTP through NMDA-dependent mechanisms and blockade of IL-1 receptors disrupts the maintenance of LTP. This evidence suggests a direct role for IL-1 in synaptic plasticity changes associated with late-LTP.

3.2.3 Neurotrophins and the Neuroimmune Response

The goal of Experiment 2 in Specific Aim 1 is to determine whether neonatal alcohol exposure (PD4-9) alters microglial activation and cytokine expression. This interest in alcohol-induce neuroimmune challenges is two-fold. First, activation of microglia and upregulation of pro-inflammatory molecules during critical periods of hippocampal development could prolong and exacerbate alcohol-induced damage and predispose these animals for cognitive and behavioral dysfunction later in life, though investigation of this last point is beyond the scope of this dissertation. Second, anti-inflammatory molecules could represent a therapeutic avenue to ameliorate deficits.

Intrinsic upregulation of these molecules would act to counter the inflammatory response and decrease production of pro-inflammatory cytokines. Infection has been shown to disrupt neurotrophin production (discussed below), meaning that anti-inflammatory cytokine signaling could have dual neuroprotective effects: downregulation of pro-inflammatory molecules and less profound reduction in neurotrophin levels.

Immune activation has been shown to reduce levels of neurotrophins in various brain regions, possibly through a negative feedback loop with the hypothalamic-pituitary-adrenal (HPA) axis (Calabrese et al., 2014). Injection of LPS significant decreased BDNF and NGF in the rat hippocampus and throughout the cortex, though the reductions were region-specific (Guan & Fang, 2006). NT-3 was only decreased in frontal cortex, suggesting a differential vulnerability of neurotrophin response to infection. Further study found decreased levels of both pro- and mature BDNF isoforms at synapses in the mouse brain, which corresponded with reductions in *Bdnf* gene expression (Schnydrig et al., 2007). Chapman and colleagues (2012) demonstrated that *E. coli* affects CA1 and dentate gyrus *Bdnf* gene expression in an exon-specific pattern, with exon I, II and IV-driven transcripts showing the largest reduction. A direct interaction between the pro-inflammatory cytokine IL-1β and BDNF was reported over two decades ago; system injection of IL-1β decreased hippocampal *Bdnf* expression, though the exact mechanism of this interaction is still unclear. While investigating the interaction between neurotrophins and cytokine expression beyond measuring each during the neonatal period is outside the scope of

the experiments conducted here, further studies are needed to clarify exactly how both infection and cytokine expression specifically impede neurotrophin production, the exact role of the HPA axis in this process, and whether the findings discussed here also hold true in the developing brain.

3.3 Exercise and Environmental Complexity Impact Hippocampal Structure and Function

Aerobic exercise and environmental complexity (referred to as environmental "enrichment" in some paradigms) are two powerful extrinsic that enhance neuroplasticity in the hippocampus through both overlapping and unique mechanisms (Olson et al., 2006). In this section, I will discuss how both of these behavioral interventions affect a number of measures related to hippocampal structure, function, and behavioral outcomes (summarized in Figure 3.2).

3.3.1 Impact of Exercise

In humans, exercise has hypothesized to promote healthy brain function since at least 1975, when Spirduso published findings that individuals engaged in high levels of activity through participation in sports had significantly faster processing speeds compared to less active individuals. Since this first study, a multitude of literature in humans and animal models has supported the beneficial effect of exercise on brain health. In humans, physical activity leads to better executive functioning and spatial and working memory performance at all ages and in both sexes (Colcombe et al., 2004; Chang et al., 2011; Erickson et al., 2009; Churchill et al., 2002). Five weeks of aerobic training in adults improved performance on a face-name recognition task

involving the medial temporal lobe (Griffin et al, 2011), and in adolescents, aerobic fitness positively correlated with ability to solve a virtual MWM task (Herting & Nagel, 2012). Data from animal models support these findings. Female non-human primates trained to run on a treadmill for one hour a day for 5 months showed faster cognitive processing on the Wisconsin General Testing Apparatus which tests learning and memory (Rhyu et al., 2010). Rodents with access to voluntary exercise had enhanced hippocampal-associated spatial memory (Marlatt et al., 2012; Vaynman et al., 2004) compared to sedentary controls. Contextual fear learning is also improved following exercise (Clark et al., 2008; Kohman et al., 2011; Schreiber et al., 2013). Finally, voluntary wheel running alleviates depressive-like symptoms in rats, which could be related to temporal lobe dysfunction (Brocardo et al., 2012).

Exercise enhances formation of LTP in the rodent hippocampus (van Praag et al., 1999; Vasuta et al., 2007; Titterness et al., 2011) and increases hippocampal volume. Both highly active adolescents and older adults have larger hippocampi, which correlates with better spatial memory performance (Herting & Nagel, 2012; Erickson et al., 2009). Even pre-pubescent children (9-10 years) with higher fitness scores had larger bilateral hippocampal volume, which correlated with better performance on an item and relation memory task (Chaddock et al., 2010). Together, these findings support that exercise is beneficial for individuals of all ages.

Exercise likely increases hippocampal volume by activation of anti-apoptotic pathways, promoting angiogenesis, and increasing adult neurogenesis (van Praag, 2009; Vivar et al., 2013; Van der Borght et al., 2009). Middle-aged female mice with

access to a running wheel for six weeks had increased labeling for VEGF, a marker of angiogenesis, in the hippocampus (Latimer et al., 2011), suggesting restructuring of the microvasculature. Similar results were found in the cortex of female non-human primates, though this study showed that regular exercise is necessary to sustain these changes to vasculature, as the changes were no longer apparent following 3 months of sedentary housing (Rhyu et al., 2010). Exercise also increases the proliferation and survival of adult-born granule cells in the dentate gyrus (Marlatt et al., 2012; Kiuchi, Lee & Mikami, 2012; Helfer et al., 2009; Hamiton et al., 2012). Furthermore, exercise promotes dendritic outgrowth and arborization (Redila & Christie, 2006; Eadie et al., 2005) and affects spine motility and density (Eadie et al., 2005; Zhao et al., 2006).

Neurotrophic factors, including BDNF, NGF, VEGF, and insulin-like growth factor (IGF) are robustly upregulated by exercise (Cotman & Berchtold, 2002; Marlatt et al., 2012; Vaynman et al., 2004; Rasmussen et al., 2009; Vivar et al., 2012; Maass et al., 2015). Griffin and colleagues (2009) found that only one week of treadmill training in rats upregulated BDNF protein levels in the hippocampus and perirhinal cortex, which correlated with improved spatial and nonspatial memory. The upregulation of BDNF by exercise has been well-studied and likely mediates many exercise-induced alterations to synaptic plasticity and adult neurogenesis (Vaynman et al., 2005). Detailed reviews of the effects of exercise on neurotrophin production and synaptic plasticity have been written by Cotman and Berchtold (2002) and the van Praag group (Vivar et al., 2012).

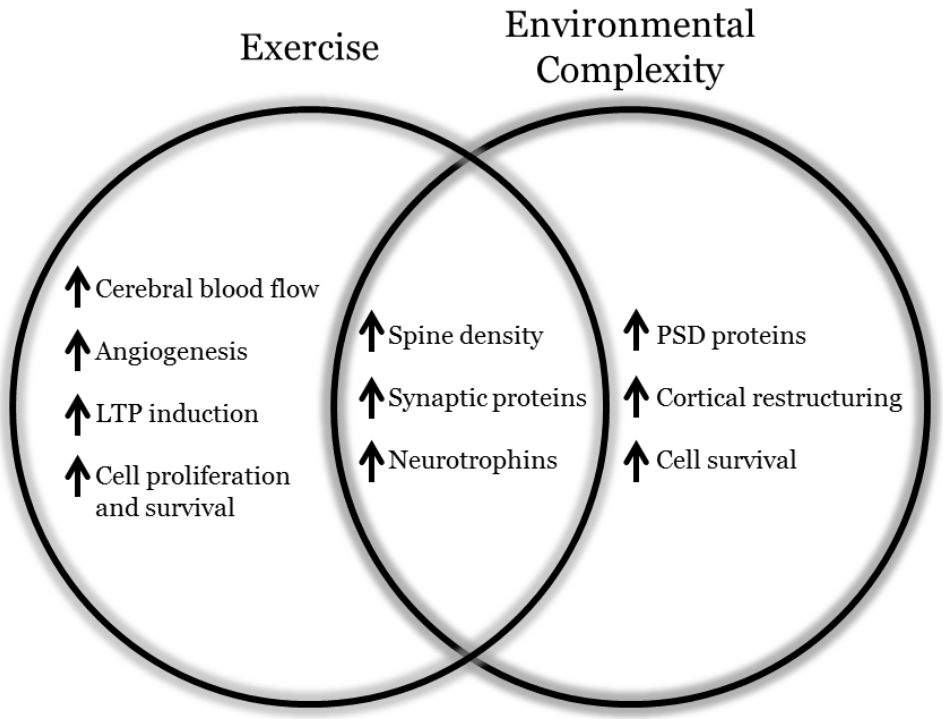

Figure 3.2. Venn Diagram Depicting the Overlapping and Unique Effects of Aerobic Exercise and Environmental Complexity on Synaptic Plasticity and Neuroanatomy.

3.3.2 Impact of Environmental Complexity (EC)

Environmental complexity (EC) paradigms vary widely, making comparisons difficult at times, but are most basically defined by "a combination of complex inanimate and social stimulations" (Rosenzweig et al., 1978). Most set ups involve a larger-than-standard cage with increased area for exploration and physical activity. The cage is filled with toys and shelter items which are usually exchanged for new objects on a regular basis, allowing for increased access to novel stimulation (Gelfo et al., 2009). In addition, these cages often house a larger number of animals to increase social interaction. Some paradigms incorporate a running wheel, particularly in the

case of mice, making interpretation of findings between paradigms even more convoluted. When discussing the relevant studies below, a short description of the paradigm used will be provided for clarity.

The Greenough group developed the "classic" EC paradigm (without a running wheel) to assess alterations to neuroanatomical and neuroplastic measures. Using this model, they published numerous landmark studies demonstrating the powerful effect of EC on dendritic morphology in the cerebellum and occipital cortex (Greenough et al., 1986; Green, Greenough & Schlumpf, 1983) and synaptogenesis in the visual cortex (Greenough et al., 1985; Turner & Greenough, 1985). Mice housed in EC show an upregulation of postsynaptic density proteins, including postsynaptic density-95 and synaptophysin (Lambert et al., 2005; Nithianantharajah et al., 2004) which are primarily found in mature dendritic spines. EC also increases brain volume, promotes restructuring and reorganization of motor cortex, and augments the number of dentate gyrus granule cells (Susser & Wallace, 1982; Walsh, 1981; Turner et al., 2003; Jha et al., 2011). Our laboratory's paradigm, which houses rats with access to a wheel for 12 days prior to 30 days in an EC cage, has demonstrated enhancement of new cell survival in the dentate gyrus (Hamilton et al., 2012) in adult rats which were exposed to alcohol during the neonatal period. Other studies (EC with and without running wheels) have concluded similarly: EC promotes the survival of adult-born neurons and has little to no effect on new cell proliferation (Kempermann et al., 1998; van Praag et al., 1999; Bruel-Jungerman et al., 2005).

Housing in EC upregulates synthesis of neurotrophins, including NT-3, NGF, and BDNF (Torasdotter et al., 1996, 1998; Pham et al., 2002; Ickes et al., 2000; Birch et al., 2013). Gobbo and O'Mara (2004) reported increased levels of BDNF in rats housed in EC (with a running wheel) and that this intervention mitigated spatial learning deficits in animals following global ischemic insult; however, EC did not provide protection against CA1 cell loss. Neuroprotection by EC (with a running wheel) against spontaneous and excitotoxic apoptosis in the hippocampus was reported by another group (Young et al., 1999). Alterations to neurotrophin production are difficult to interpret in many of these studies due to the inclusion of a running wheel in the paradigm. Thus, it is not known if these increases are driven by aerobic exercise, the novelty and social interaction components, or a combination of these factors.

As I have alluded to in the previous paragraph, evidence supports that the beneficial effects of exercise and EC on behavior and cognition likely arise from both unique and overlapping mechanisms (summarized in Figure 3.2). Recent work has suggested that access to running wheels within EC cages is the driving factor in the observed upregulation of adult neurogenesis and neurotrophin levels (Kobilo et al., 2011; Mustroph et al., 2012). Housing mice with access to novelty items but no wheel did not show the enhanced neurogenesis or BDNF levels that were present in animals in the EC+running condition. However, not all studies have supported this claim. A study by Faherty and colleagues (2003) housed mice in an EC cage (with a wheel) and demonstrated more complex dendritic morphology in multiple cell types (CA1

pyramidal cells, dentate gyrus granule cells, striatal spiny neurons). Interestingly, this enhancement was only found in mice housed in the EC cage, not in mice with access only to a running wheel, suggesting that some other component of the EC cage was strongly influencing dendritic outgrowth.

While paradigms which include EC (not overlapping with, but *following* access to voluntary exercise) have shown changes to behavioral outcomes (Schreiber et al., 2013; Hamilton et al., 2011), EC has not been shown to affect LTP, microvasculature or cerebral blood flow (Olson et al., 2006; Goldman et al., 1987). In fact, while many of the synaptic markers required for LTP induction are upregulated by EC (Tang et al., 2001), this paradigm can reverse previously established LTP (Abraham et al., 2002). However, even a reversal of LTP indicates synaptic plasticity is occurring within the circuit, even if the function of this reversal is unclear.

3.4 Conclusions

Neurotrophic factors have a critical role in hippocampal development and neuroplasticity. Additionally, microglia and cytokine expression are important to synaptic maintenance and normal cognitive function in adulthood. Activation of microglia during brain development could contribute to hippocampal dysfunction and alter how the brain responds to later immune challenges. The behavioral interventions of wheel running and EC both enhance neuroplasticity and impact hippocampal structure and function in overlapping and/or complementary manners, representing potential behavioral therapies developmentally alcohol-exposed animals during adolescence or adulthood. Chapter 4 will further discuss these topics, specifically in

regards to the impact of neonatal alcohol exposure on adult neurogenesis, dendritic complexity, BDNF production, and neuroimmune activation, and our work supporting implementation of wheel running and housing in a complex environment as promising therapeutic avenues for alcohol-related deficits. Finally, I will discuss analysis of *Bdnf* DNA methylation as a novel approach to assessing changes to regulation of BDNF levels following neonatal alcohol exposure.

Chapter 4

FOUNDATIONAL STUDIES

This dissertation aims to examine the short-term and long-term effects of postnatal alcohol exposure on BDNF levels in the hippocampus of neonatal and adult rats. It will also investigate the impact of postnatal alcohol exposure on microglial activation and cytokine levels in the neonatal hippocampus. This dissertation will also examine whether neonatal alcohol exposure affects the process of adult neurogenesis and dendritic morphology of immature neurons in the adult rat dentate gyrus. Finally, it will explore the possible therapeutic influence of aerobic exercise and housing in a complex environment on BDNF, adult neurogenesis, and dendritic morphology. Prior to discussing the experiments, it is important to review the existing literature to develop an experimental basis for the questions that will be asked and addressed in this dissertation. This chapter reviews the most pertinent studies which serve as a foundation for the experiments I present in my dissertation[3].

4.1 Neonatal Alcohol Exposure Negatively Impacts Hippocampal Adult Neurogenesis and Dendritic Morphology

Adult neurogenesis is the process through which the brain continues to generate new neurons throughout the lifespan in two specific regions: the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of

[3] Sections of text in this chapter have been reproduced or adapted with permission from Klintsova, Hamilton & Boschen (2013) and Boschen & Klintsova, *in press*.

the hippocampal dentate gyrus. Cells from the SVZ migrate via the rostral migratory stream to the olfactory bulb where they mature into interneurons, while most proliferating cells in the SGZ differentiate into dentate gyrus granule cells and migrate into the granule cell layer and integrate fully into the hippocampal trisynaptic pathway. This process is thought to be important for behaviors and cognition associated with the hippocampus, including spatial learning, context memory, pattern separation and completion, and emotion regulation. Performance on tasks that involve these dimensions of learning are often impaired in animal models of developmental alcohol exposure, and humans with FASDs often have significant learning disabilities.

Adult neurogenesis is separated into stages of cell generation and maturation: 1) proliferation, 2) differentiation, 3) migration, 4) maturation, and 5) long-term survival (dependent on successful synaptic integration). The entire process from cell birth to full integration takes up to three months. Alcohol exposure during early development can impact both the health of neural progenitors in the adult rodent brain and the ability of these newly born granule cells to successfully mature and integrate into the hippocampal trisynaptic circuit, though the most consistent effects are reported on neuronal survival. Prenatal alcohol exposure decreased the size of the progenitor pool and number of proliferating neurons in one report (Redila et al., 2006) and a single binge on PD7 was shown to disrupt cell proliferation (Ieraci & Herrera, 2007). However, several other studies did not demonstrate an effect of alcohol on cell proliferation (Choi et al., 2005; Klintsova et al., 2007; Gil-Mohapel et al., 2011; Hamilton, et al., 2011).

Deficits in neuronal maturation processes and survival caused by developmental alcohol exposure have been shown repeatedly (Klintsova et al., 2007; Gil-Mohapel et al., 2011; Hamilton et al., 2011; Ieraci & Herrera, 2007; Singh et al., 2009), suggesting that alcohol exposure, particularly during the brain growth spurt period, permanently alters signaling pathways or gene expression necessary for successful maturation and synaptic integration. Multiple papers from our laboratory have focused on this phenomenon. In 2007, our group demonstrated that PD4-9 binge-like alcohol exposure (5.25 g/kg/day) decreased the survival of newly generated cells in the adult rat hippocampus. Rats were injected with bromodeoxyuridine (BrdU) which labels actively proliferating cells going through the "S" phase of cell division every other day from PD30-50. On PD50, no change in the number of BrdU+ cells (proliferating cells) was seen; however, when brains were assessed 30 days following the injections (PD80), there were significantly fewer cells surviving in the alcohol-exposed group compared to controls. This reduction in surviving cells was replicated 30-34 days following single injections on PD42 or 80 (Hamilton et al., 2011, 2012, 2014). Additionally, we housed the rats in 12 days of wheel running (PD30-42) followed by 30 days of standard housing (PD42-72) and found that while exercise increased new cell proliferation in alcohol-exposed animals on PD72, this group still had fewer surviving neurons after 30 days of standard housing compared to controls (BrdU injections on PD41; Figure 4.1) (Hamilton et al, 2012). In all these experiments, no alcohol-induced change in proliferation was observed using either the exogenous marker BrdU or the endogenous protein Ki-67.

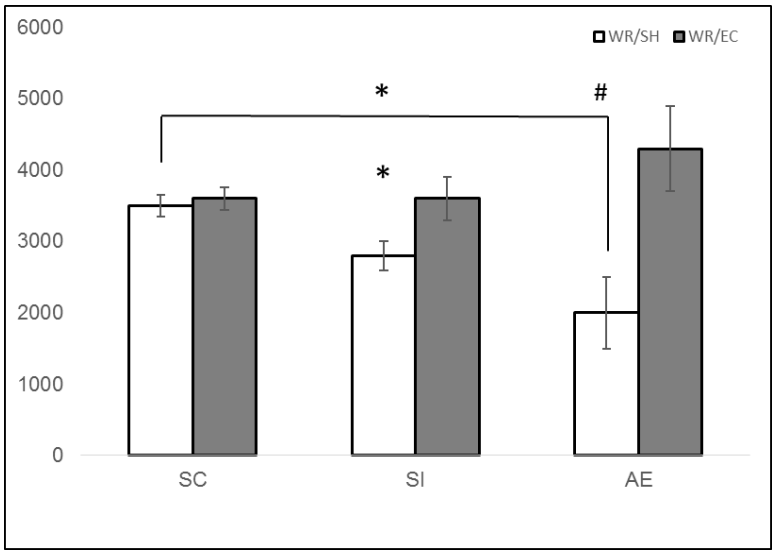

Figure 4.1. "PD72 Analysis of BrdU Labeling Using Light Microscope.

Images of BrdU+ labeled cells recognized by reaction with diaminobenzidine and counterstained with Pyronin Y. Images taken at 20×. (A) is taken from an AE animal exposed to WR/EC. (B) is taken from an AE animal exposed to WR/SH. (C) demonstrates that the number of BrdU+ cells is significantly decreased in AE animals compared to SC when exposed to standard housing after running ($p<0.05$). Exposure to environmental complexity significantly increases the number of surviving new cells in both the SI ($p < 0.05$) and the AE ($p < 0.01$) groups compared to social housed littermates. All values represent mean +/− SEM. * $p < 0.05$; # $p < 0.01$." Figure adapted with permission from Hamilton et al., 2012.

The number of immature neurons has also been measured using the marker doublecortin, which labels a population of neuronal progenitors and immature neurons ranging from a few days to two weeks old. PD4-9 alcohol exposure did not influence the number of doublecortin-labeled cells in the adult rat brain. Thus, it is likely that the reduction in neuronal survival in alcohol-exposed animals takes place soon after the cells have stopped expressing doublecortin. Ultimately, an inability of the hippocampus to produce and integrate the number of new granule cells necessary for

new memory formation could contribute to the cognitive deficits in children with FASDs and the behavioral impairments observed in animals models.

Developmental alcohol exposure has deleterious effects on dendritic morphology and spine density in late-developing structures such as the prefrontal cortex and hippocampus. The exact mechanism through which developmental alcohol exposure compromises dendritic complexity in the adult brain is not well understood, but it is thought that these alterations contribute significantly to cognitive deficits in children with FAS. In fact, in cases of mental retardation due to either genetic or environmental factors, “dendritic abnormalities are the most consistent anatomical correlates” reported (Kaufman & Moser, 2000). Perinatal alcohol exposure (combining pre- and postnatal exposure) had limited effect on dendritic structure in the nucleus accumbens, but reduced spine density on Layer II/III pyramidal cell dendrites in the mPFC (Lawrence et al., 2012). Multiple studies from our lab have shown PD4-9 alcohol exposure to have a negative impact on medial prefrontal cortex dendritic complexity. Specifically, our model of FASD decreased basilar dendritic complexity in Layer II/III pyramidal neurons of the mPFC (Hamilton et al., 2010; 2015). Spine morphology on the basilar dendrites also shifted to a more mature, less plastic phenotype, though spine density was unchanged. On the other hand, the apical dendrites are affected in an almost opposite pattern by this model of alcohol exposure: dendritic tree morphology was stable but spine density was significantly decreased in alcohol-exposed animals (Whitcher & Klintsova, 2008). Similar results were found following PD2-6 exposure via vapor inhalation: Layer II/III basilar dendrites had a

simplified structure compared to controls (Granato et al., 2003). Based on these findings, it is possible that prefrontal cortex communication with subcortical structures is impaired in alcohol-exposed animals.

Alcohol-induced alterations to dendritic complexity in hippocampus have also been reported and this literature could give valuable clues as to why adult-born dentate gyrus granule cells do not have the same survival rate as in the healthy brain. Administration of alcohol to cultured CA1 pyramidal cells decreased dendritic length and number of dendrites per cell (Yanni & Lindsley, 2000). Spine density of CA1 neurons is not affected by prenatal alcohol in rats housed in isolation; however, alcohol-exposed rats housed in a complex "enriched" environment (which increases spine density in normal animals) display no change in spine density (Berman et al., 1996). These finding suggest that 1) timing of the alcohol exposure could dictate whether baseline changes to dendritic complexity and spine density are present and 2) that deficits might not be apparent until the system is challenged in some way. In the dentate gyrus, chronic alcohol exposure in adult rats decreased dendritic length of granule cells (Carneiro et al., 2008). Shorter exposures (1-4 weeks) also decreased dendritic complexity of immature neurons in the adult dentate gyrus (He et al., 2005). Specifically, the number of dendritic endings and total length was decreased following all alcohol exposure paradigms. Impaired dendritic outgrowth of immature neurons could directly dictate their ability to make sufficient synaptic connections and successfully integrate into the hippocampal network and cause them to undergo apoptosis. Additionally, changes to dendritic morphology of mature neurons could be

indicative of disrupted hippocampal connectivity and circuit dysfunction. Overall, the abnormalities in dendritic morphology and spine density in alcohol-exposed animals likely contribute significantly to the behavioral deficits observed in rodent models of FASDs.

4.2 Neurotrophins as Targets of Alcohol Exposure

Alterations to neurotrophin signaling are one pathway through which developmental alcohol exposure could negatively affect adult neurogenesis and dendritic morphology. Alcohol during development can have direct consequences on neurotrophin signaling, even long after cessation of the exposure. The effects of alcohol exposure on neurotrophin and receptor expression are highly dependent on a number of variables, including the alcohol dose and developmental window targeted, timing of the tissue collection following the exposure, route of administration, and the brain region in question. Interstudy variability on these factors can make the literature difficult to parse. The most well-studied neurotrophin-alcohol relationship is the interaction with BDNF and its receptor TrkB, though numerous papers have assessed NGF as well. NT-3 and VEGF have been less explored, though it is likely that more resources will be put towards investigating VEGF in more detail in the future due to an emerging understanding of its role in neuroplasticity.

In recent years, numerous studies have been published trying to determine how developmental alcohol exposure affects BDNF levels in the brain, and often these studies have been at odds with one another due to the range of alcohol exposure paradigms, rodent species, and timing of the tissue analysis utilized, as well as

differences in which brain regions were examined. While there is little doubt that BDNF expression is altered following rodent models of FASD, questions remain regarding the directionality and stability of the changes. It is not known if alcohol interacts with the BDNF molecule or TrkB receptor directly; it seems more likely that BDNF levels are affected through action of alcohol on NMDA or $GABA_A$ receptors. For example, in cerebellar granule cell cultures, BDNF was increased following NMDA treatment, however this enhancement was blocked following pretreatment with ethanol (Bhave et al., 1999).

Models of prenatal alcohol exposure have consistently demonstrated changes to BDNF signaling in various brain regions. Following exposure from gestational day 5-20, BDNF protein and mRNA were reduced in the rat cortex and hippocampus when assessed on PD7-8 (Feng et al., 2005). Mice exposed to alcohol prenatally show decreased levels of BDNF protein, total and exon III, IV and VI-driven *Bdnf* mRNA transcripts in adulthood in the mPFC (Caldwell et al., 2008); no changes in protein were found in hippocampus in these mice but they did exhibit downregulation of certain exon-driven mRNA transcripts. Another study found that at 18 months of age, levels of BDNF were depleted in the liver and elevated in the hippocampus of prenatally exposed mice, suggesting an interaction with developmental alcohol exposure and natural aging processes (Ceccanti et al., 2012). Prenatal exposure also has been shown to alter levels of the TrkB receptor, inhibiting the phosphorylation of TrkB on PD7-8 while leaving the total amount of TrkB unchanged (Feng et al., 2005). In another study, male rat pups exposed to alcohol *in utero* also showed decreased

levels of TrkB receptors on postnatal day 1 in the hippocampus, no changes to TrkB in the septum or cerebellum, and increased levels in cortex (Moore et al., 2004). Interestingly, female pups showed a different pattern of changes, with levels of TrkB decreased in septum, no changes in hippocampus, and consistent increases in TrkB in cortex. Most alterations to receptor number had returned to baseline by postnatal day 10. These studies highlight the variety of changes to BDNF signaling which can vary by brain region, sex of animal, and time point. In general, the consensus of these prenatal studies seems to point to decreased BDNF production in hippocampus following prenatal alcohol exposure, with more variability reported in other brain regions.

BDNF and TrkB receptor expression have also been shown to be altered in postnatal alcohol exposure models. Studies assessing BDNF 24 hours or less following neonatal exposure have found increased protein levels in hippocampus and cortex (PD2-10 or PD4; Heaton et al., 2000, 2003) and decreased *Bdnf* and *Trkb* gene expression in cerebellum (PD2-3 and PD4 exposures; Light et al., 2001; Heaton et al., 2003). Another report assessing *Bdnf* mRNA and downstream signaling pathways on PD8 following PD5-8 alcohol exposure found decreased levels of *Bdnf* and downregulation of the MAPK and Akt pathways in the cerebral cortex (Fattori et al., 2008). Postnatal exposure (PD10-15) via vapor inhalation disrupted normal age-related fluctuations in *Bdnf* gene expression, with alcohol-exposed rats having higher expression in the hippocampus on PD16 and 20 and decreased expression on PD60 compared to control animals, again supporting an interaction between alcohol

exposure and age on alterations in neurotrophin signaling (Miki et al., 2008). Aside from age-related fluctuations, BDNF production is also linked to the circadian cycle. Allen and colleagues (2004) reported that PD4-9 alcohol exposure significantly decreased overall levels of BDNF protein in the suprachiasmatic nucleus (SCN) and blunted the circadian rhythmicity of expression when the animals were 5-6 months of age. BDNF is thought to contribute to circadian regulation through action in the SCN (Liang et al., 2000) and disruptions to circadian rhythms have been reported in both children with FASDs and in rodent neonatal exposure models (Chen et al., 2012; Sakata-Haga et al., 2006).

This dissertation will use a lifespan approach to determine whether neonatal alcohol exposure (PD4-9) alters short-term and long-term hippocampal BDNF expression by analyzing tissue at a neonatal (PD10) and an adult (PD72) time point. In addition, my dissertation seeks to expand on previous literature by investigating BDNF at multiple levels at each time point: BDNF protein, *Bdnf* total and exon-specific gene expression, and *Bdnf* DNA methylation. Ultimately, this approach will give new information regarding how our model of FASD affects BDNF production at the protein and molecular levels.

4.3 Neurotrophins as Neuroprotective Molecules in the Developing Brain

BDNF and other neurotrophins have been long-investigated for their role in neuroprotection, with some evidence supporting the investigation of neurotrophins as compensatory molecules during or following developmental alcohol exposure (Pezet & Malcangio, 2004; Davis, 2008; Idrus & Thomas, 2011). As discussed in Chapter 2,

alcohol exposure induces widespread waves of apoptosis throughout the developing brain shortly following administration. BDNF prevents apoptosis in a variety of experimental paradigms, including excitotoxicity and ischemic hypoxia (Almeida et al., 2005; Han et al., 2000). There is also evidence that pharmacological modulation of NGF and BDNF can protect against cytotoxicity and apoptosis following alcohol exposure, though this effect has only been directly demonstrated *in vitro*. Both BDNF and NGF reduced cytotoxicity as measured by a reduction in 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in a model of ethanol exposure combined with hypoxia and hypoglycemia (Mitchell et al., 1999). Administration of alcohol to cultured human neuroblastomas decreased cell viability (mitochondrial activity) and BDNF and CREB levels; exogenously applied BDNF improved mitochondrial activity in alcohol-exposed cells (Sakai et al., 2005). Alcohol induced apoptosis in cerebellar granule cells *in vitro* 12-24 hours after administration, but the upregulation of cell death was blocked by co-administration of BDNF (Bhave et al., 1999). Finally, Climent and colleagues (2002) showed that BDNF activated anti-apoptotic and pro-cell survival pathways in the postnatal cerebral cortex; however, this activation is disrupted by prenatal alcohol exposure. Further research needs to be done to determine if these results hold true *in vivo*. Numerous studies have found enhanced levels of neurotrophins in the hippocampus and cortex within 24 hours of alcohol administration, suggesting a possible endogenous mechanism of neuroprotection (Heaton et al., 2000, 2003). This enhancement may be timing- and region-specific and temporally restricted, with possible links to alcohol withdrawal. It is not known if

blocking this upregulation of neurotrophins would further alcohol-related cell loss or which isoform of BDNF protein is increased (pro vs. mature BDNF).

While administration of exogenous neurotrophic factors to cultured cells has shown some therapeutic promise, little work has been done exploring administration of neurotrophins to alcohol-exposed rodents, possibly due to logistical or technical issues (Davis, 2008). The neuroprotective effect of neurotrophic factors would depend largely on timing of administration following the alcohol exposure. To achieve true protection from the negative effects of alcohol, the neurotrophin would be administered either shortly before or during the alcohol exposure, or possibly during the withdrawal period. BDNF infused intracerebroventricularly to mice exposed prenatally to alcohol and stress has positive benefits on anxiety-like behavior (marble burying) and aberrant sexual and social interactions (Popova et al., 2011), however these effects were only apparent following 7-10 days of daily infusions. Incerti and colleagues (2010) reported that embryos exposed to alcohol on GD8 had decreased levels of BDNF after 6 hours and enhanced levels after 24 hours; BDNF levels were normalized with neuropeptides related to vasoactive intestinal peptide (VIP). Administration of these neuropeptides in adulthood reversed alcohol-induced learning deficits (Incerti et al., 2010). VIP and BDNF have been shown to reciprocally enhance expression of one another in certain cell types (Pellegri et al, 1998; Cellerino et al., 2003). Recently, other pharmacological agents including peroxisome proliferator-activated receptor-γ (PPAR-γ), which suppresses production of pro-inflammatory cytokines (Kane et al., 2015), and lithium (Luo, 2010) have shown promise in

protecting against alcohol-induced cytotoxic damage. Lithium, for one, is known to enhance neurotrophin levels, and activation of the neuroimmune response can downregulate BDNF expression, calling for future research to determine if these drugs exert action, at least in part, through neurotrophin signaling.

4.4 Neonatal Alcohol Exposure Induces the Neuroimmune Response

Children with *in utero* exposure to alcohol have a higher risk of developing secondary health complications, such as respiratory illnesses, in part due to increased likelihood of premature birth and nutritional deficiencies in alcohol-exposed infants (Gauthier, 2015). Recent work in animal models has begun to delve into the complex neuroinflammatory response induced by developmental alcohol exposure and investigation of this response as a secondary source of damage during alcohol exposure and withdrawal. To date, most *in vivo* work has focused on postnatal FASD models and the short-term impact of alcohol on markers of neuroinflammation. PD4-9 exposure in a mouse model of FASD increases microglial activation and pro-inflammatory cytokine gene expression (IL-1β, TNF-α, CCL2, CCL4, CD11b) in the hippocampus, cerebellum, and cortex on PD10 (Drew et al., 2015). PD3-5 alcohol vapor inhalation also induced pro-inflammatory cytokine gene expression in the hippocampus and cerebellum of the neonatal pups, with particular upregulation of cytokine expression during withdrawal periods (Topper et al., 2015). Ethanol administration to cultured cerebellar microglia has potentially neurotoxic effects, reducing the number of microglia while simultaneously increasing activation and production of pro-inflammatory cytokines in the remaining cells (Kane et al. 2011).

Tiwani and Chopra (2011) showed that aberrant activation of the developing immune system can have long-term consequences on cytokine expression. PD7-9 alcohol exposure via intubation resulted in enhanced levels of TNF-α and IL-1β in the cerebral cortex and hippocampus in adulthood. This finding is important as aberrant activation of the immune system early in development could result in overactivation of microglia and overproduction of inflammatory molecules following further immune challenges later in life and contribute to pathological conditions (Bilbo & Schwarz, 2009); overexpression of cytokines at baseline conditions in the alcohol-exposed brain could indicate disruption of normal immune function in these animals. Interestingly, there is limited evidence that postnatal alcohol exposure might also enhance levels of cytokines with anti-inflammatory properties. Levels of TGF-β mRNA were increased in the adult cortex and hippocampus following PD7-9 exposure (Tiwani & Chopra 2011). However, Topper and colleagues (2015) reported no changes to TGF-β or another anti-inflammatory cytokine, IL-10, in the neonatal brain following PD3-5 vapor inhalation; these differences could be due to the model or timing of exposure used. Further investigation of how anti-inflammatory cytokines might react to alcohol-induced damage in the developing brain is needed and considered as a potential therapeutic intervention to mitigate apoptosis.

4.5 Behavioral Interventions Reverse Hippocampal Dysfunction in Rodents Models of FASDs

In rodent models of FASD, exercise and environmental complexity have shown promise as therapeutic treatments for a wide range of alcohol-related deficits,

with particularly robust effects found on hippocampal anatomy, plasticity, and behavioral measures. This section will discuss relevant literature showing how exercise and environmental complexity can reverse hippocampal dysfunction in alcohol-exposed animals. In particular, I will detail studies conducted by the Klintsova lab.

Exercise can enhance measures of synaptic plasticity in alcohol-exposed animals, including LTP in the perforant pathway in prenatally exposed animals (Christie et al., 2005), hippocampal expression of immediate early gene c-Fos, a marker of neuronal activation (Sim et al., 2008), and expression of BDNF (Boehme et al., 2005). Notably, exercise interventions for models of FASD have been shown to increase new cell proliferation in the dentate gyrus (Boehme et al., 2005; Redila et al., 2006; Helfer et al., 2009). Conflicting results have been found for exercise's effect on new cell survival in alcohol-exposed animals. A study from our laboratory (Helfer et al., 2009) found that 30 days of social housing following 12 days of voluntary exercise was not sufficient to enhance cell survival in neonatally (PD4-9) alcohol-exposed animals, while survival was increased in control animals. However, Boehme and colleagues (2005) have reported a robust benefit in cell survival 28 days after 12 days of wheel running in animals exposed through all three trimester-equivalents. Reasons for these conflicting results could be the sex or genetic strain of the animals used, as well as differing developmental time points for wheel running access (early vs. late adolescence) and tissue analysis. Wheel running during adolescence also increased

pyramidal cell spine density and dendritic length in the medial prefrontal cortex of neonatally exposed rats (Hamilton et al., 2015).

Aerobic exercise has been shown to enhance performance on hippocampal-associated tasks of spatial memory. Alcohol-exposed (either prenatal or postnatal exposure) rats have been reported to display impaired spatial memory on the Morris Water Maze task (Christie et al., 2005; Thomas et al., 2008). However, extended access to a running wheel shortened the latency to the platform in the alcohol-exposed group to a level indistinguishable from controls. Perinatally alcohol-exposed animals also show increased anxiety- and depression-like symptoms as measured on an elevated plus maze, an open field test, and a forced swim test (Brocardo et al., 2012). Twelve days of wheel running showed mixed impact on these symptoms, and some of the effects differed based on sex on the animal. Male alcohol-exposed rats showed anti-anhedonic effects of wheel running in the forced swim test as evidenced by less time spent immobilized in the water; conversely, female rats did not show this benefit. No positive effect was shown in the open field or elevated plus maze. Thomas and colleagues (2008) also reported increased locomotion in the open field test in neonatally alcohol-exposed rats; this overactivity was ameliorated by 30 days of wheel running during adolescence. Overall, the above studies support that exercise is a potential behavioral therapy for individuals with FASD, particularly if the intervention given is in childhood or adolescence.

Our laboratory employs two behavioral interventions: 42 days of wheel running access or 12 days of wheel running followed by 30 days housed in a complex

environment (WREC). This latter intervention, termed the “super-intervention”, has proven beneficial to alcohol-exposed rats on numerous measures of neuroplasticity. Our model of FASD (PD4-9 exposure, 5.25 g/kg/day via intragastric intubation) decreases the survival of adult-born dentate gyrus granule cells. Specifically, animals were injected with BrdU on PD41, which is the day before rats in WREC are changed from their WR condition to EC. Cell survival (BrdU+) and proliferation (Ki-67+) was assessed 30 days later on PD72. Housing in WREC rescues new cell survival in alcohol-exposed animals back to control levels (Hamilton et al., 2012, 2014). Further, this intervention reverses the alcohol-induced decrease in basilar dendritic complexity in Layer II/III pyramidal cells in medial prefrontal cortex (Hamilton et al., *in preparation*). On a behavioral level, housing in WREC enhances contextual fear and trace eyeblink conditioning in alcohol-exposed rats (Hamilton et al., 2011; Schreiber et al., 2013; Hamilton et al., 2014). Interestingly, WREC did not improve social behavior deficits observed in alcohol-exposed animals (Boschen et al., 2014). Exploratory and social behavior of male rats was recorded during their housing in the EC cage. Alcohol-exposed animals explored less of the cage and engaged in fewer mounting and wrestling behaviors compared to controls. While housing in EC allows the animals increased exposure to novelty and opportunities for social interactions, this condition did not serve as an intervention on these behavioral measures. It is possible that our intervention has more influence on hippocampal-associated tasks and plasticity, while social behavior is heavily reliant on the amygdala and prefrontal cortex.

4.6 *Bdnf* Gene Methylation as a Novel Approach to Assess Long-Lasting Changes to BDNF in the Hippocampus.

One avenue through which neonatal alcohol exposure might impact *Bdnf* gene expression is via epigenetic alterations. Epigenetics refer to chemical modifications to DNA and histones that can influence gene transcription independent of alterations to the nucleotide sequence. Two of the most common epigenetic modification are histone acetylation and DNA methylation, the latter of which is the focus of this dissertation. DNA methylation refers to the addition of methyl groups to cytosines by DNA methyltransferases to for 5-methylcytosine (Blaze & Roth, 2015). Methylation occurs largely at the cytosines of a CpG dinucleotide (Figure 4.2). Promoter regions of genes are often enriched with CpG sites which have dynamic methylation patterns and methylation of these sites can affect downstream gene transcription. DNA methylation usually has the effect of silencing gene expression, as methyl groups can either interfere with the binding of transcription factors necessary to promote gene transcription and/or bind Methyl-CpG Binding Protein 2 (MeCP2) which can recruit repressor proteins such as histone deacetylases (HDACs) that promote a compact chromatin state not permissive to gene transcription (Roth et al., 2009; Law & Jacobsen, 2010).

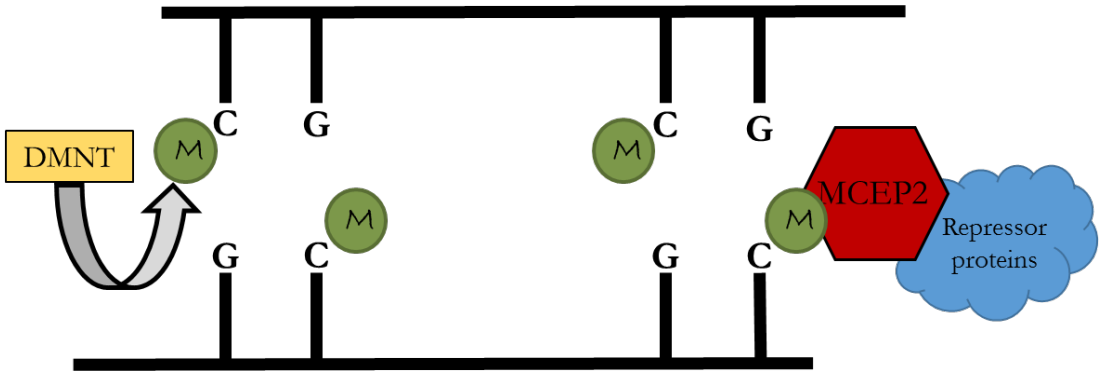

Figure 4.2. Schematic of DNA Methylation.

C: cytosine, G, guanine, M: Methyl group, DMNT: DNA methyltransferase, MeCP2: Methyl-CpG Binding Protein 2.

Recent studies have shown that aversive early life experiences can alter *Bdnf* DNA methylation both transiently and permanently (dependent upon specific *Bdnf* gene locus) in the hippocampus and prefrontal cortex (Roth et al., 2009; Blaze et al., 2013). In regards to early-life exposure to alcohol and DNA methylation alterations, embryos exposed *in vitro* to alcohol during neurulation had altered DNA methylation for genes associated with key developmental processes (Liu et al., 2009), altered histone methylation and acetylation, and increased activity of DNA methyltransferases (Veazey et al., 2015). *In vivo*, methylation of the proopiomelanocortin (POMC) gene was increased in the hypothalamus in adult mice following prenatal alcohol exposure along with other epigenetic histone modifications (Govorko et al., 2012). Global methylation was increased on PD21 in the hippocampus of rats exposed to alcohol PD2-10 (Otero et al., 2012) and perinatal alcohol exposure increased hippocampal DNA methyltransferase activity when measured at PD21, which could result in hypermethylation of DNA (Perkins et al., 2013). This dissertation seeks to determine

if *Bdnf* shows altered DNA methylation in the hippocampus following neonatal alcohol exposure. If *Bdnf* DNA methylation patterns are affected across development, then it is possible that downstream protein expression would be affected and, in turn, BDNF-associated neuroplasticity

Both exercise and environmental complexity decrease hippocampal *Bdnf* DNA methylation in healthy adult animals (Gomez-Pinilla et al., 2011; Kuzumaki et al., 2011). Exercise also increased levels of MeCP2 and BDNF mRNA and protein levels (Gomez-Pinilla et al., 2011). In some cases, MeCP2 can bind proteins such as CREB which enhance gene expression (Blaze & Roth, 2015), though it's not known if this was the case in this particular study. If *Bdnf* DNA methylation is increased in adult rats neonatally exposed to alcohol, this evidence suggests that either our lab's behavioral interventions of exercise alone or exercise followed by housing in a complex environment might potentially reverse these alterations. Changes to *Bdnf* DNA methylation caused by exercise and/or environmental complexity would be a newly discovered pathway through which these behavioral interventions enhance plasticity in the damaged brain.

4.7 Conclusions

In this chapter I have reviewed the most relevant studies which form the experimental basis for this dissertation. Specifically, I have shown that developmental alcohol exposure disrupts adult neurogenesis in the hippocampal dentate gyrus, induces dendritic abnormalities, targets neurotrophin production, and activates microglia and pro-inflammatory cytokine production. I have also supported the

investigation of neurotrophins as innate compensatory molecules and exercise and environmental enrichment as extrinsic behavior therapies to protect against alcohol-induced tissue damage and apoptosis. Finally, I have argued for the importance of investigating *Bdnf* DNA methylation as a novel approach to understanding how neonatal alcohol exposure impacts neurotrophin signaling. Before moving on to discuss the experiments in this dissertation, I will first introduce the general techniques and methods used in my Specific Aims (Chapters 6-8) in the next chapter.

Chapter 5

GENERAL PROCEDURES

This chapter describes the general methods used in the experiments presented in this dissertation. The methods are based on published studies (Hamilton et al., 2012; Boschen et al., 2014, 2015, 2016). Permission for reuse of text was obtained where appropriate.

5.1 Subjects

Timed Long-Evans pregnant dams were obtained from Harlan Laboratories (Indianapolis, IN) and housed in standard cages (17 cm high x 145 cm long x 24 cm wide) in a 12/12 h light cycle (lights on at 9:00 AM) upon arrival. On postnatal day (PD) 3, litters were culled to eight pups each (6 male, 2 female when possible to control for single-sex litter effects on maternal care and preweaning pup interaction). Pups were marked using subdermal injections of non-toxic black ink on their paws to indicate individual pup number within the litter. 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 SI and AE animals were represented in the same litter. Following the alcohol exposure procedure, pups were left undisturbed with the dam until sacrifice (PD10) or weaning (PD23). In total, 231 male rat pups were generated for the current study (43 litters). Separate cohorts of animals were generated for molecular biology (ELISA, gene expression, DNA methylation) and immunohistochemical assays. The specific sample sizes for used for

each experiment are listed in the methods of that particular study. All procedures were carried out in accordance with NIH Animal Care Guidelines and the animal use protocol approved by University of Delaware Institutional Animal Care and Use Committee.

5.2 Alcohol Exposure

On PD4-9, alcohol-exposed (AE) pups were exposed to alcohol in a binge-like manner (5.25 g/kg/day) via intragastric intubation using a well-established method in our lab (Klintsova et al., 2007; Helfer et al., 2009). This alcohol dose mimics heavy binge drinking during the third trimester of human pregnancy (Bonthius & West, 1990). Alcohol was administered in an 11.9% v/v milk solution in two doses, two hours apart (9:00 and 11:00 AM). Briefly, a P20 polyethylene tube fitted to the end of a blunted needle is attached to a liquid-filled syringe. The tapered tip of the tube is dipped in corn oil and gently maneuvered into the mouth of the pup, down the esophagus and into the stomach. To minimize the potential for puncturing of the stomach, the tube length is measured prior to insertion into the mouth by measuring the distance from the mouth to the milk spot on the pup's stomach. The dose of alcohol/milk or milk-only solution is then slowly injected into the stomach. On PD4, two supplemental doses of milk formula were administered, two and four hours following the second alcohol dose as a caloric supplement for the AE pups. For PD5-9, milk formula was administered only once, two hours following the second alcohol exposure. A total of 10 male pups died during or shortly following the intubation procedure. Sham-intubated (SI) pups act as a stress control for the intubation procedure and are intubated in the manner described above but did not receive any milk or alcohol solution during the intubation procedure. Delivering milk to SI pups has been previously demonstrated to result in significant weight gain above the norm

(Goodlett & Johnson, 1997). Suckle control (SC) animals were left undisturbed with the dam apart from daily weighing (PD4-9) at 9 AM.

5.3 Blood Alcohol Concentration (BAC) Analysis

On PD4, 90 minutes following the second alcohol exposure, blood samples were obtained from AE pups via a small tail clip for BAC analysis. Blood was collected in a 70 µl heparinized capillary tube which was then emptied into a 1.5 ml microcentrifuge tube. Samples from the AE group were centrifuged (15,000 rpm/15-25 minutes at 4°C) and the plasma collected and stored at -20ºC. Plasma was analyzed for BAC through enzymatic reaction using an Analox GL5 Alcohol Analyzer (Analox Instruments, Boston, MA). Blood samples were also taken from SI group at the same time to control for stress of the blood collection procedure, but were not analyzed for BACs.

5.4 Adolescent and Adult Housing Conditions

Weaning occurred on PD23 and rats were placed in standard cages with 3 same-sex animals per cage, counterbalanced across litter and neonatal condition. In most cases, the cage contained 1 AE, 1 SI, and 1 SC animal, all from different litters. The rats remained in these cages until PD30, when each cage was assigned one of three adolescent/adult housing conditions: standard social housing (SH), continuous access to wheel running for 42 days (WR/WR), or 12 days of wheel running access followed by 30 days of housing in a complex environment (WR/EC). The animals were housed in these conditions and weighed approximately every 8 days until sacrifice on PD72.

5.4.1 Wheel Running (WR)

Rats were housed in cages with 24 h voluntary access to stainless steel running wheels (Figure 5.1). The wheel was attached to an opaque, standard sized cage via a hole through which one adult-sized rat could easily pass. Animals were housed 3 per cage in the same configuration as PD23-30 housing (counterbalanced for litter and neonatal condition). The total running distance per 24 h period was determined by the number of wheel revolutions registered by a mechanical counter attached to each of the wheels and recorded daily at 9 AM. Cages were cleaned every 4th day at 9 AM. As animals were housed 3 per cage, the exact distance run by each rat could not be recorded. Previous studies from our lab housed AE, SI and SC animals separately in the wheel running condition (3/cage) and found no effect of neonatal treatment on distance run per day (Helfer et al., 2009). The rats are also regularly observed running together in the wheel. Based on these observations, wheel running totals are analyzed by cage, not by individual rat.

Figure 5.1. Example of a Cage with Wheel Running Access.

A standard-sized cage which houses 3 animals is attached to a stainless steel running wheel for 24 h voluntary access. A mechanical counter on the side of the wheel tracks the number of rotations.

5.4.2 Environmental Complexity (EC)

Housing in EC occurred between PD42-72, with the rats being transferred from cages with wheel access to an EC cage at 9 AM on PD42. Each EC cage consisted of a 30” x 18” x 36” 3-story galvanized steel cage with three ramps, two balconies and a full middle floor (Figure 5.2). The floor of the cage was a drop-in 3½ - inch plastic pan filled with wood chip bedding to allow for digging. Each cage housed 9-12 male animals (3-4 animals/neonatal condition) and was equipped with a variety of toys and novelty objects, such as wiffle balls, stacking rings, toy keys, and plastic

blocks. Large tubes and buckets were placed in the cage as shelter. The toys were changed at 9:00 AM every 2nd day. Every 4th day at 9:00 AM, the cages were cleaned by removing all the animals (~10 minutes), replenishing food, water and bedding, and replacing all of the toys with novel items.

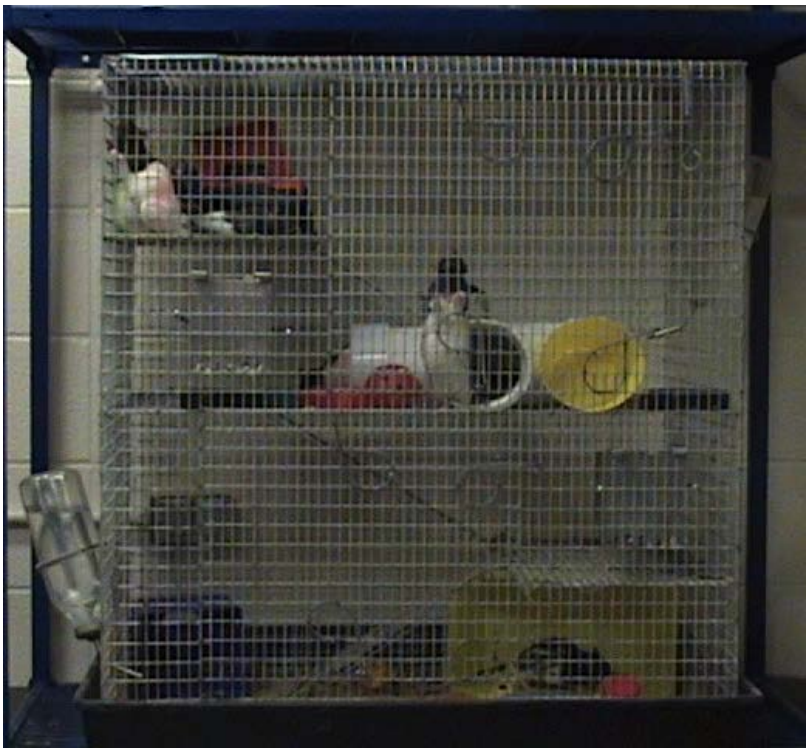

Figure 5.2. Example of a Complex Environment (EC).

Animals are housed 9-12 per EC cage with access to toys which are exchanged for novel items every other day.

5.5 Immunohistochemistry

On day of sacrifice, animals were taken directly from their housing condition to be euthanized. On PD10 or PD72, animals were deeply anesthetized (ketamine/xylazine cocktail), transcardially perfused (0.1M PBS with heparin

followed by 4% paraformaldehyde) and the brains stored in 4% paraformaldehyde for 24 hours. The brains were then transferred into 30% sucrose in 4% paraformaldehyde for 2-3 days, then transferred to fresh 30% sucrose in 4% paraformaldehyde for another 2 days. For PD10 tissue, this procedure was repeated once more. Brains were sectioned coronally or horizontally at 40 µm through the entirety of the hippocampus and collected in wells maintaining the order. Immunohistochemistry was performed on a pseudo-randomly selected set of section ($1/8^{th}$, $1/12^{th}$ or $1/16^{th}$) which included the entirety of the hippocampus or dentate gyrus only (Specific Aim 3). The specific immunohistochemical procedures used for each primary antibody will be described in the appropriate experiment's methods section. For all experiments, two control sections per animal were processed identically to the immunolabeled sections but were placed in blocking solution only instead of primary antibody; staining of these sections insured antigen-specific labeling. Sections were then mounted onto gelatinized slides and allowed to dry overnight. The sections were then counterstained with Pyronin Y and dehydrated with ethanol before being coverslipped using DPX mounting medium. Slides then dried for one week before analysis.

5.6 Analysis of Immunolabeled Hippocampal Tissue

5.6.1 Cell Count Estimates Using Unbiased Stereology

Cell quantification was performed in an unbiased stereological manner using the optical fractionator probe (Stereo Investigator, Micro Bright Field Inc., Williston, VT). Specific parameters (e.g. grid size) used for each experiment will be described in appropriate methods section. Briefly, beginning with the dorsal-most (horizontal) or

anterior-most (coronal) section, the entire hippocampus (Aim 1) or dentate gyrus only (Aim 3) was analyzed. The section-sampling fraction was 1/8, 1/12 or 1/16, depending on the experiment and the section thickness fraction was the ratio of the dissector height (12 μm) to the mean thickness of the sections, measured using the software at every 5th counting frame. Slides were coded so that the experimenter was blind to treatment condition and counts were made within a known volume of the hippocampal region of interest.

5.6.2 Microglial Cell Territory Analysis

As an indirect estimate of microglia morphology and activation state, total cell area or "territory" (Drew et al., 2015) was measured using NeuroLucida software (v. 10.52, MBF BioScience, Williston, VT). For each section, the area encompassed by an individual microglia was measured by tracing a contour from the tip of each microglial process to the next while measuring through the depth of the section (Figure 5.3). Five pseudorandomly selected microglia were traced per section from microglia located within the granule (dentate gyrus) or pyramidal (CA1, CA3) cell layers or immediately adjacent with at least one process extending into the cell layer. The areas of the microglia were the averaged within and across sections. Activated microglia are usually characterized by shorter, thicker processes and a larger soma whereas resting microglia have longer, thinner processes, meaning that smaller microglia cell territory would indicate a more activated morphology.

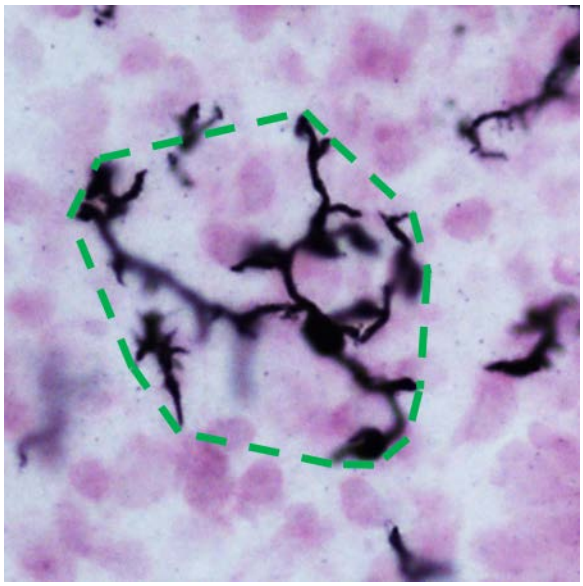

Figure 5.3. Tracing Around the Processes of a Microglia to Determine Cell Territory Measurement.

5.6.3 Dendritic Complexity Analysis

Immature dentate gyrus granule cells (doublecortin (DCX) positive) were traced and Sholl analysis conducted using a computer-based neuron tracing system (NeuroLucida v. 10.52; MBF, Bioscience, Williston, VT). The experimenter remained blind to animal condition throughout the analysis procedure. Two sections of dorsal dentate gyrus were used per animal for the analysis. For each animal, the experimenter identified fully labeled DCX+ granule cells at 400x magnification (40x objective). All cell bodies were located in the dentate gyrus or subgranular zone and included in the analysis if they met criteria: 1) fully labeled DCX+ cell in postmitotic stage (presence of a dendrite extending towards the molecular layer (Plumpe et al., 2006)), 2) dendritic tree contained in the analyzed section, and 3) the dendritic tree's branches were not broken or obscured. These criteria allowed for 6-10 cells to be traced per animal,

which is consistent with previous work from our lab tracing Golgi-Cox-impregnated medial prefrontal cortex Layer II/III pyramidal cells (Hamilton et al., 2010, 2015; Whitcher & Klintsova, 2008). NeuroLucida software was used to conduct a Sholl analysis of each neuron (Figure 5.4). Branching order was automatically determined by the software starting at the first dendritic bifurcation. The Sholl analysis placed the center of the neuron at the center of the cell's soma and then drew 20 μm radians extending out from this point. The number of intersections at each radius, total dendritic length per radius and number of nodes/bifurcations per radius was determined, which allowed for analysis of dendritic complexity. The average length of each cells' dendritic tree, average number of intersections, length per radius, and number of bifurcations per cell were also analyzed for each animal.

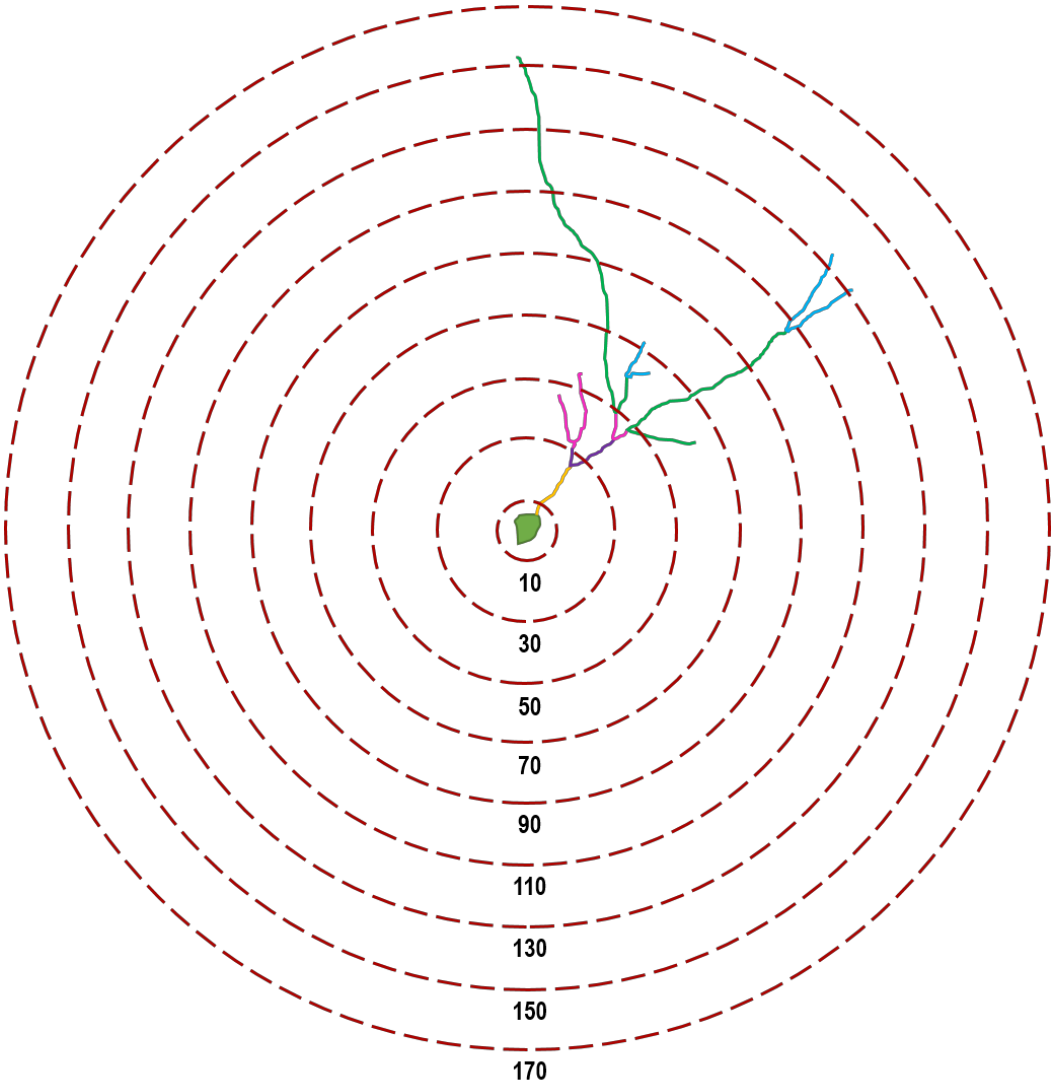

Figure 5.4. Sholl Analysis was used to Determine Dendritic Complexity.

Radii extended from the center of the soma at 20 µm intervals beginning with 10 µm.

5.7 Gene Expression Analysis

Animals were rapidly decapitated and the brains frozen with -20ºC 2-methylbutane in a brain matrix. Razor blades were used to cut the brain into 1 mm coronal sections which were mounted onto slides and stored at -80°C until processing. Dorsal and ventral hippocampus were dissected on dry ice, combined, and homogenized in RLT Plus lysis buffer (Qiagen, Valencia, CA) and 2-betamercaptoethanol. The homogenized tissue was store at -80°C until the DNA and RNA were extracted using the AllPrep DNA/RNA Mini Kit (Qiagen). DNA and RNA were stored at -80°C. Quantification and analysis of nucleic acid quality were performed with spectrophotometry (NanoDrop 2000, ThermoScientific, Waltham, MA). cDNA was synthesized by reverse transcription using the Quantitect Reverse Transcription Kit (Qiagen) and stored at -20°C. cDNA was amplified with real-time polymerase chain reaction (RT-PCR) (Bio-Rad, CFX96) using the appropriate primers and mixes for each reaction. The specific primers and master mixes used will be described in the appropriate methods section for each experiment. Following amplification, the fold change for each target gene relative to the SC (PD10) or SC/SH (PD72) group was determined using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

5.8 Protein Expression Analysis

BDNF, TrkB, and corticosterone protein levels were analyzed using Enzyme-Linked Immunosorbent Assay (ELISA). BDNF and TrkB were analyzed from hippocampal tissue and corticosterone was measured from plasma. Animals were sacrificed via rapid decapitation on PD10 (24 h after the last alcohol exposure) and

conditions of the animals were counterbalanced across the day to account for possible diurnal changes in corticosterone and BDNF. Hippocampus from one hemisphere (both dorsal and ventral, left/right counterbalanced) was removed from each animal, weighed, and stored at -20℃ until lysation. During sacrifice, trunk blood was collected for corticosterone assays. Samples were kept on ice until they were centrifuged at 4ºC for 25 minutes, after which plasma was collected and stored at -20℃.

5.8.1 Enzyme-Linked Immunosorbent Assay (ELISA)

Hippocampal tissue was homogenized in 4X cell lysis buffer (20mM Tris-HCL [pH 7.5], 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1µg/ml leupeptin; Cell Signaling Technology) and 1X protease and phosphatase inhibitor cocktail (ThermoScientific). The final concentrations of the homogenized hippocampus was 1:8. The lysate was then centrifuged (25 min at 15,000 rpm at 4℃) and the supernatant collected for analysis. Total brain protein was detected with Coumassie Protein Assay (ThermoScientific). Tissue and plasma were diluted and processed according to the manufacturer's specifications and suggestions for each kit, which will be listed in the appropriate experiment's methods section. All plates were read using a spectrophotometer (Tecan Infinite F50). Data were expressed as pg target protein per mg of total brain protein for tissue and pg corticosterone per mL for plasma. Results were then combined as percent of control between plates.

5.9 DNA Methylation Analysis

DNA from the total hippocampus (dorsal and ventral) was extracted using AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA) from the same animals used for the *Bdnf* gene expression analysis. *Bdnf* exon I DNA methylation status was characterized based on alcohol-specific effects on exon I-specific gene expression on PD10. Methylation status was assessed via methylation-specific PCR (MSP) on bisulfite-converted DNA. Bisulfite conversion was performed using the Epitect DNA kit (Qiagen) starting with a concentration of 25 ng/µl of DNA. Conversion was performed using the recommended parameters in a mini thermal cycler. Following conversion, the DNA was cleaned using manufacturer provided spin columns and reagents and stored at -20°C until amplification.

Bisulfite-converted DNA was amplified by primers that targeted the methylated and unmethylated *Bdnf* exon I promoter region using polymerase chain reaction (PCR), including the transcription start site. *Tubulin* was used as a reference gene. The primer sequences have been previously published (Roth et al., 2009) and are listed in Chapter 6, Table 1. All reactions were run in triplicate. Following amplification, the fold change for both methylated and unmethylated DNA relative to the SC (PD10) or SC/SH (PD72) group was determined using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). The methylation index for each animal was determined by dividing the fold change for the methylated primer set by the fold change for the unmethylated primer set.

5.10 Statistical Analyses

For neonatal studies (Specific Aim 1), BACs and body weights from PD4, 9 and 10 were collected. Weights were analyzed with a repeated-measures Analysis of Variance (ANOVA) with the independent variables of Day x Neonatal Condition. For each day, a one-way ANOVA was also performed to determine differences in neonatal weights between conditions on each day. Protein levels, microglial cell number, and cell territory were analyzed using one-way ANOVAs (independent variable: Neonatal Condition) with appropriate *post hoc* analysis (Tukey's). Gene expression and methylation status were assessed using Wilcoxon Signed Rank test or one-sample *t*-test against the hypothetical value of 1 (SC). Unpaired *t*-tests were used to determine differences between the AE and SI groups. Specific analyses and details regarding data presentation are described for each experiment.

For adult studies (Specific Aims 2 and 3), BACs, body weights (PD4, 9, 30, 42, 72), and running activity were collected and analyzed for each subject. Neonatal weights and adolescent/adult weights were analyzed separately. For neonatal weights, a repeated-measures ANOVA was run with the independent variables of Day x Neonatal Condition. A one-way ANOVA was also performed to determine differences in neonatal weights between conditions on PD4 and 9 separately. For PD30, 42, and 72, a repeated-measures ANOVA (Day x Neonatal Condition x Housing) was run. For Aim 2, gene expression and methylation status were assessed using a two-way ANOVA (independent variables: Neonatal Condition x Housing) with appropriate *post hoc* tests (Tukey's). For Aim 3, planned comparisons (one-way ANOVAs followed by

Tukey's *post hoc* test) were run to test for differences between individual neonatal or housing conditions for Ki-67+ and DCX+ counts and dendritic complexity measures, based on previous work from our lab (Hamilton et al., 2015, *in preparation*). Specifically, within the SH condition, the three neonatal treatments were compared. Then, within each neonatal treatment, the effect of housing condition was assessed. For the Sholl analysis, the variables of average length per radius, average intersections per radius, average bifurcations per radius, average total dendritic length per cell, average bifurcations per cell, and average number of intersection per cell were analyzed for each animal. Specific analyses and details regarding data presentation are described for each experiment.

GraphPad Prism 6 or the SPSS (v14) statistical package were used for all analyses. Grubb's test was used to identify and remove outliers from each data set. Statistical significance was determined to be a $p < 0.05$; trends of $p < 0.1$ are indicated as well.

Chapter 6

SPECIFIC AIM 1: IMPACT OF NEONATAL ALCOHOL EXPOSURE ON BDNF EXPRESSION AND THE NEUROIMMUNE RESPONSE IN THE DEVELOPING RAT HIPPOCAMPUS

6.1 Introduction

Alcohol exposure during the third trimester-equivalent (first two postnatal weeks in rodent models; Dobbing & Sands, 1979) has a significant impact on the structure and function of various brain regions forming during this time, including the hippocampus and prefrontal cortex. During this sensitive time window, known as the brain growth spurt, these brain regions undergo critical developmental changes, including massive neurogenesis, synaptogenesis, cell migration, and gliogenesis. Studies using rodent models of FASD have demonstrated that third trimester-equivalent alcohol exposure causes waves of apoptosis of post-mitotic cortical and hippocampal neurons (Ikonomidou et al., 2000; Olney et al., 2002), in addition to long-lasting reductions in hippocampal CA1 pyramidal cell number (Tran et al., 2003; Livy et al., 2003), impaired induction of CA1 long-term potentiation (LTP) (Puglia et al. 2010a,b), decreased neuronal activation as measured by expression of the protein c-Fos (Murawski et al., 2012), impaired survival of newly generated dentate gyrus granule cells (Klintsova et al., 2007; Hamilton et al., 2010, 2012), and alterations to mature granule cell morphology (Gil-Mohapel et al., 2011). Targeting molecular changes immediately following alcohol exposure might prevent long-term brain damage.

6.2 Experiment 1: Impact of Neonatal Alcohol Exposure on BDNF and TrkB Protein Levels, *Bdnf* Exon-Specific Gene Expression, and *Bdnf* DNA Methylation in the Developing Rat Hippocampus[4]

One potential pathway through which developmental alcohol exposure might affect short- and long-term neuroplasticity is via alterations to levels of brain-derived neurotrophic factor (BDNF). BDNF is critical for normal cellular maturation processes, including proliferation, migration, dendritic arborization, synaptogenesis, and induction and maintenance of LTP (Chan et al., 2008; Tolwani et al., 2002; McAllister et al., 1997; Lu et al., 1999; Hall et al., 2000; Alonso et al., 2002; Heldt et al., 2007). Developmental alcohol exposure has been shown to alter BDNF protein levels in various brain regions, including the hippocampus, prefrontal and motor cortex (Heaton et al., 2000; Heaton et al., 2003; Feng et al., 2005; Barbier et al., 2008; Caldwell et al., 2008; Fattori et al., 2008); though, the nature of these effects (i.e. increases or decreases) are dependent on alcohol dose, exposure window, brain region, and timing of analysis, suggesting a developmental time-specific vulnerability. Most studies have assessed either only BDNF protein or mRNA expression, making the outcomes incomplete and comparison across studies difficult. Additionally, investigating expression of BDNF's high-affinity tyrosine kinase receptor tropomyosin-related kinase B (TrkB) in the same paradigm is necessary to better

[4] Experiment 1 text and figures (aside from DNA methylation data and discussion) used with permission from Boschen et al., (2015).

understand possible cellular mechanisms of aberrant plasticity after developmental alcohol exposure. Receptor expression or function might also be affected by developmental alcohol exposure, potentially influencing downstream signaling pathways involved in neuroplasticity and learning and memory.

Epigenetic modifications, which refer to the addition or deletion of chemical groups on chromatin (DNA and its associated histone proteins) that do not affect the DNA sequence itself, have recently come to the forefront as a mechanism through which environmental factors could directly impact genes and, in turn, behavior. DNA methylation, one of the most widely studied epigenetic modifications, is the addition of methyl groups to cytosines at CpG dinucleotides in the promoter region of genes. Methyl groups on these CpG sites can bind transcriptional repressor proteins, such as MeCP2, and recruit histone deacetylases (HDACs), resulting in decreased transcription of the gene. A number of environmental toxins induce alterations to DNA methylation status, including bisphenol A (BPA) and arsenic (Kundakovic & Champagne, 2011; Reichard & Puga, 2010). Models of developmental alcohol exposure cause a variety of epigenetic modifications dependent on the timing of the alcohol administration (Liu et al., 2009; Govorko et al., 2012; Otero et al., 2012; Perkins et al., 2013), however investigation of gene-specific epigenetic modifications caused by developmental alcohol exposure is still a largely unexplored field. Methylation of *Bdnf* is altered in timing- and exon-specific manner following a variety of early life experiences, including prenatal stress and postnatal caregiving environment (reviewed in Blaze & Roth, 2015). DNA methylation can not only affect

baseline gene expression, but can also prime changes to gene transcription in response to environmental stimulation (Baker-Andresen et al., 2013). Thus, alcohol-induced changes to *Bdnf* methylation status in the early postnatal period could inform both alterations to *Bdnf* gene expression at this time point, but modifications to transcriptional regulation later in life.

Various routes of administration are used in models of developmental alcohol exposure, including intraperitoneal injection, alcohol vapor inhalation, maternal voluntary drinking paradigms, and administration via intragastric gavage of the dam or pups (reviewed in Patten et al., 2014). The suitability of each method is based on the timing, dose and pattern of alcohol exposure being modeled. The current study employs intragastric intubation of the pups from postnatal days 4-9 to mimic alcohol exposure during the third trimester of human pregnancy. Intubation allows for precise dosing of each pup based on individual weight and absorption of the alcohol through the gastrointestinal tract, while also achieving high, binge-like blood alcohol concentrations (300-400 mg/dl using our model [Hamilton et al., 2012; Boschen et al., 2014]). Since the intubation procedure is quite invasive and stressful for the pups, a sham-intubated group is used as a stress control. These animals are intubated without administration of any liquid for the same duration and number of times as the alcohol-treated pups. Previous work has shown that sham-intubation does not increase cortical and cerebellar apoptosis or cell loss in the same manner as alcohol exposure (Goodlett et al., 1998; Bonthius et al., 2001; Green et al., 2002; Tran et al., 2003) and is regarded as less stressful compared to previously used postnatal models (e.g., artificial rearing

or “pup in a cup” method described in West, 1993; Dominguez & Thomas, 2008). In recent years, the role of stress in early development has been demonstrated to be detrimental on a wide variety of neuroplasticity measures, making the consideration of stress in the current intubation paradigm more essential than ever. The impact of intubation stress on subtle neuroanatomical and behavioral measures is critical for the understanding of this method and researchers’ continuing mission to develop models which most closely mimic the human condition.

The current experiments investigate whether third trimester-equivalent binge-like alcohol exposure or intubation stress alone (PD4-9) affect BDNF and TrkB receptor protein levels, *Bdnf* gene expression, and *Bdnf* DNA methylation in the hippocampus of infant rats (PD10). Additionally, as we were interested in exploring the degree of stress caused by the intubation method and how this stress might affect experimental findings, we measured plasma corticosterone levels on PD10. This study broadens our knowledge regarding whether developmental alcohol exposure and intubation stress similarly and differentially affect neurotrophic factor production during infancy and provides important information to researchers using a similar alcohol exposure model concerning the effect on glucocorticoids.

6.2.1 Materials and Methods

For detailed information regarding the subjects, materials and methods used, see Chapter 5 (General Procedures). Methods will be briefly described below with details pertaining to the specific experiments in this Aim.

6.2.1.1 Subjects

Briefly, timed pregnant Long-Evans dams were obtained from Harlan Laboratories (Indianapolis, IN). On postnatal day (PD) 3, litters were culled to eight pups each (6 male, 2 female when possible to control for effects of having a single-sex litter). On PD4, pups were randomly assigned to one of three experimental groups: suckle control (SC), sham-intubated (SI) or alcohol exposed (AE). Following the alcohol exposure procedure, pups were left undisturbed with the dam until sacrifice (PD10). Due to the capricious nature of the TrkB Enzyme-linked Immunosorbant Assay (ELISA) kit, some tissue was lost due to kit failure, resulting in a need for increased litters of animals to be generated for this assay. In total, 84 male rat pups were generated for the current study (from a total of 16 litters). The specific sample sizes for each experimental condition used for each assay are listed on the x-axis of each figure.

6.2.1.2 Alcohol Exposure and BAC analysis

On PD4-9, AE pups were exposed to alcohol in a binge-like manner (5.25 g/kg/day) via intragastric intubation (Figure 6.1A). A total of 6 male pups died during or shortly following the intubation procedure. On PD4, blood samples were obtained from AE pups via tail clip for BAC analysis 90 minutes following the second alcohol exposure. Plasma was analyzed for BAC using an Analox GL5 Alcohol Analyzer (Analox Instruments, Boston, MA).

6.2.1.3 Protein Expression Analysis

Animals were sacrificed via rapid decapitation on PD10 (24 h after the last alcohol exposure; Figure 6.1A) and trunk blood collected for corticosterone assays. Kits used for each analysis were Chemikine BDNF Sandwich ELISA kit (Millipore, Billerica, MA), Rat Neurotrophic Tyrosine Kinase Receptor Type 2 ELISA kit (NovateinBio, Woburn, MA), and Corticosterone ELISA kit (Enzo Life Sciences, Farmingdale, NY). Total brain protein was detected with Coumassie Protein Assay (ThermoScientific). Data were expressed as pg target protein per mg of total brain protein for tissue and ng corticosterone per mL for plasma. Results were then combined as percent of control between plates.

6.2.1.4 Gene Expression Analysis

The hippocampus (both dorsal and ventral) was dissected on dry ice and homogenized. DNA/RNA were extracted using the AllPrep DNA/RNA Mini Kit (Qiagen Inc., Valencia, Calif., USA). Reverse transcription was performed using a cDNA synthesis kit (Qiagen) on RNA, and cDNA was amplified by real-time PCR (Bio-Rad CFX96) with Taqman probes (Applied Biosystems) to target *Bdnf* total mRNA (exon IX) and exon I- and IV-specific transcripts. These specific regions of the *Bdnf* gene were chosen because they are highly sensitive to environmental manipulations (Sathanoori et al., 2004; Aid et al., 2007; Nair et al., 2007; Roth et al., 2009). *Tubulin* was used as a reference gene. All reactions for each gene target and reference were run in triplicate. Product specificity was verified using gel electrophoresis.

6.2.1.5 DNA Methylation Analysis

Methylation status of *Bdnf* exon I was assessed using methylation specific real-time PCR (MSP) on bisulfite-converted DNA (Qiagen Inc., Valencia, CA). Exon I was chosen due to the alcohol-specific effect on gene expression for this region of the *Bdnf* gene. Primer sets (Sigma-Aldrich; listed below in Table 1; Roth et al., 2009; Roth et al., 2015) targeted methylated and unmethylated CG dinucleotides in DNA associated with *Bdnf* exon I or *tubulin* (used as a reference gene). All reactions were run in triplicate. Primer specificity was determined by melt curve analysis on all samples and gel electrophoresis on a sub-set of samples.

Primer	Sequence (5' to 3')
***Bdnf* exon I Methylated**	Forward: CGGAAAGTATTAGAGGTAGGGTAGC Reverse: TACGAACCCTAAATCTCTAAACGAA
***Bdnf* exon I Unmethylated**	Forward: TGGAAAGTATTAGAGGTAGGGTAGTGA Reverse: TACAAACCCTAAATCTCTAAACAAA
Tubulin	Forward: GGAGAGTAATATGAATGATTTGGTG Reverse: CATCTCAACTTTCCCTAACCTACTTAA

Table 6.1. Custom Primer Sets for MSP Analysis.

6.2.1.6 Statistical Analyses

Weights for each day of dosing (PD4-9) and day of tissue harvest (PD10) were averaged across neonatal condition per day and analyzed using a repeated-measures analysis of variance (ANOVA) followed by post hoc tests when appropriate. For blood alcohol concentrations, average PD4 BACs for each animal (two or three analyses run per animal) ± standard error of the mean (SEM) are reported as mg/dl. For BDNF and

TrkB protein data, pg target protein/mg total brain protein data were transformed into percent of control between plates to control for differences in the ELISA kit standards. Inconsistent optical density values (>30% difference between duplicate wells) were removed from the analyses. Differences in BDNF and TrkB protein data were analyzed using one-way ANOVAs with appropriate *post hoc* tests run as needed (Tukey's). For gene expression and MSP data, the comparative Ct method was used to obtain the relative fold change of experimental (AE or SI) vs. the average of controls (SC) per plate (Livak and Schmittgen, 2001). Comparisons between the experimental groups were performed using unpaired *t*-tests and comparisons between the experimental (AE and SI) and the control group (SC) were analyzed with a Wilcox Signed Rank Test (gene expression) or one-sample t-test (MSP) (hypothetical value set to1.0). A mean value of 1 would indicate no change in transcript level in comparison to the suckle control group. For plasma corticosterone data, an independent-samples Kruskal–Wallis test was run to account for unequal variance between groups with *post hoc* pairwise comparisons. Differences were considered to be statistically significant at $p < 0.05$ and nonsignificant trends at $p < 0.1$ are also reported. For all studies, outliers were identified using Grubb's test. All statistical analyses were run using Prism 6 software (GraphPad Inc.).

6.2.2 Results

6.2.2.1 Body Weights and BACs

All neonatal treatment groups gained weight throughout the treatment period. A two-way ANOVA revealed a significant interaction between postnatal day and

neonatal treatment ($F_{(12, 567)} = 2.413$, $p < 0.01$; Figure 6.1B). In addition, there were main effects of both postnatal day ($F_{(6,567)} = 556.3$, $p < 0.001$) and neonatal treatment ($F_{(2, 567)} = 78.66$, $p < 0.001$). While animals from all neonatal treatments did not differ in weight on PD4 (means ± SD: SC: 10.9g ±1.2, SI: 10.9g±0.7, AE: 10.8g±0.7), Tukey's HSD *post hoc* test revealed that AE animals weigh significantly less starting on PD5 ($p < 0.05$, Figure 6.1B). By PD10, AE animals weighed an average of 2 g less compared to both SC and SI (SC: 22.9g±2.7, SI: 22.4g±1.4, AE: 20.1g±1.7). Similar decreases in AE weights have been reported using this model of alcohol exposure. The effects on weight are temporally limited, as we have shown in other studies that AE animals do not differ in weight from SC and SI rats when assessed on PD30 (Hamilton et al., 2012; Boschen et al., 2014). However, the role of under-nutrition should not be discounted when interpreting the results of these studies.

AE animals had an average BAC concentration of 366.4 mg/dl (± 84.1 SD) on PD4, 90 min following the second alcohol dose. This value is consistent with previous work published from our lab using the same alcohol exposure paradigm (Hamilton et al., 2012, Boschen et al., 2014).

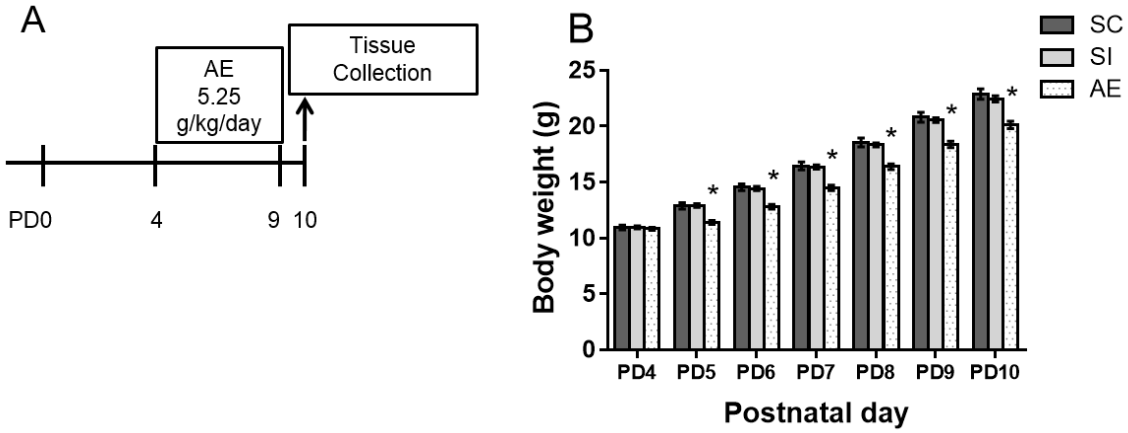

Figure 6.1. Experimental Timeline and Body Weights.

A) On PD 4-9, rats were exposed to 5.25 g/kg/day ethanol via intragastric intubation and sacrificed on PD10. AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control. B) Average body weight ± SEM are shown for each day during the neonatal treatment period (PD4-9) as well as the day of sacrifice for alcohol-exposed (AE), sham-intubated (SI), and suckle controls (SC). AE animals weighed significantly less than SI and SC animals on PD5-10. * $p < 0.05$. Image reprinted with permission from Boschen et al., 2015.

6.2.2.2 BDNF and TrkB Protein Levels

BDNF protein levels were measured from PD10 hippocampal tissue. A one-way ANOVA revealed a main effect of neonatal condition ($F_{(2,36)} = 4.833$, $p = 0.014$, Figure 2A). *Post hoc* tests (Tukey's HSD) showed that AE pups had significantly increased BDNF protein compared to the SC group ($p = 0.016$), while SI showed marginally increased BDNF levels (vs. SC, $p = 0.074$) but did not differ significantly from AE pups (p = 0.9). TrkB protein levels were also measured from PD10 hippocampal tissue. A one-way ANOVA revealed a main effect of neonatal condition ($F_{(2,34)} = 4.875$, $p = 0.014$, Figure 2C) showing that AE pups had significantly higher

TrkB protein levels compared to SI animals (p = 0.01), but not from SC animals (p = 0.12).

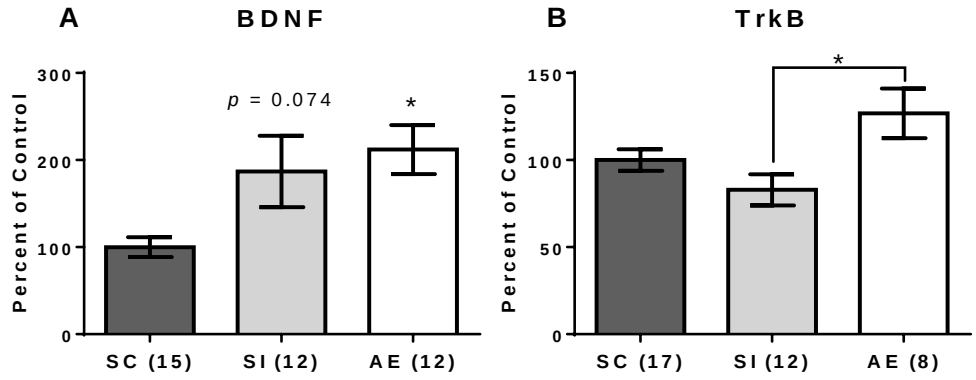

Figure 6.2. BDNF and TrkB Protein in the Hippocampus on PD10.

A) AE animals had significantly more BDNF protein than SC animals. SI pups showed a trending increase in BDNF vs. the SC group (p = 0.074) but were not significantly different from AE pups. B) AE animals had more TrkB protein in the hippocampus relative to SI, but not SC pups. * p < 0.05. Data is shown as mean percent of control ± SEM. AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control. Image reprinted with permission from Boschen et al., 2015.

6.2.2.3 *Bdnf* Exon-Specific Gene Expression

Total *Bdnf* gene expression (by targeting all exon-IX containing transcripts) was measured in the hippocampus on PD10. Total *Bdnf* gene expression was increased in both the AE and SI groups compared to the SC group on PD10 (p = 0.019 and 0.034 for AE and SI vs. SC, respectively; Figure 6.3A), and a *t*-test revealed that the AE and SI groups did not differ from one another (p = 0.28). We also examined exon I- and IV-specific transcripts as expression of these mRNAs are vulnerable to early-life experiences (Sathanoori et al., 2004; Nair et al., 2011). For exon I-specific transcripts, only AE pups had significantly increased gene expression compared to SC pups (p =

0.027; Figure 6.3B), but did not significantly differ from SI (p = 0.29). For exon IV-specific transcripts, gene expression was significantly increased in AE animals (p = 0.027; Figure 6.3C) and there was a trending increase found in the SI group (p = 0.09). AE and SI groups did not differ from one another (p = 0.90).

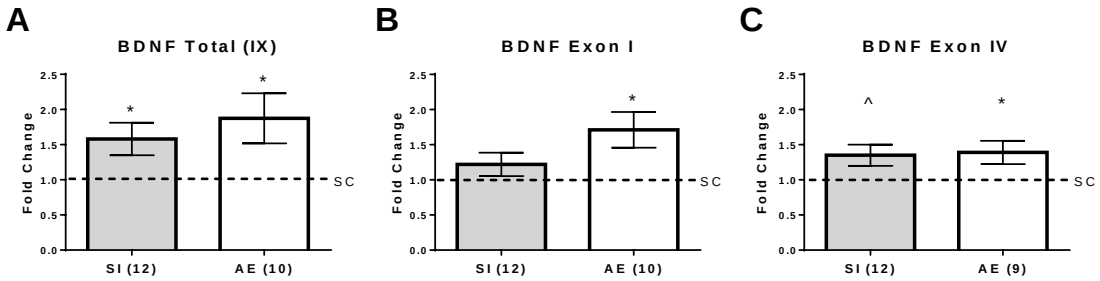

Figure 6.3. *Bdnf* Gene Expression in the PD10 hippocampus.

A) AE and SI animals had higher total *Bdnf* gene expression vs. SC. B) and C) Exon I- and IV-specific mRNA was significantly elevated in AE pups. SI pups showed a trending increased in exon IV mRNA vs. SC (p = 0.09, indicated as ^ on graph). * p < 0.05. Data is expressed as a fold change ± SEM from the SC group (shown as 1). AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control. Image reprinted with permission from Boschen et al., 2015.

6.2.2.4 *Bdnf* Exon I DNA Methylation

DNA methylation status of the *Bdnf* exon I promoter region was assessed via MSP in the hippocampus on PD10 (Figure 6.4). There was less methylation associated with *Bdnf* exon I DNA methylation in both the AE and SI groups compared to the SC group on PD10 (p = 0.0124 and 0.0005 for AE and SI vs. SC, respectively). A *t*-test revealed that methylation levels in the AE and SI groups were similar (p = 0.9478).

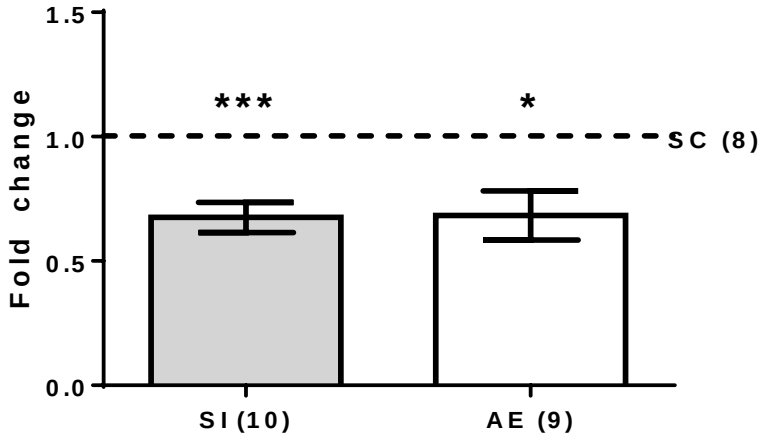

Figure 6.4. *Bdnf* Exon I DNA Methylation in the PD10 Hippocampus.

AE and SI animals showed significantly less *Bdnf* DNA methylation at exon I in the hippocampus compared to SC. * $p < 0.05$, *** $p < 0.001$. Data is expressed as a fold change ± SEM from the SC group (shown as 1 on graphs). AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control.

6.2.2.5 Plasma Corticosterone Levels

Corticosterone was measured from PD10 plasma to assess the role that stress might play in the current results. Trunk blood was collected at the time of sacrifice for all generated animals, making it possible to analyze corticosterone levels for all the cohorts generated for these experiments. An independent-samples Kruskal-Wallis test revealed a main effect of neonatal treatment ($\chi^2(2) = 13.576$, $p < 0.001$; Figure 6.5A), with AE and SI animals showing significantly increased corticosterone levels compared to the SC group ($p < 0.001$ and $p = 0.045$, respectively) but not differing from one another ($p = 0.924$). These results support the use of sham intubation as a stress control for the alcohol dosing procedure. For reference, average raw corticosterone levels were ~50 ng/ml plasma for the SC group (Figure 6.5B), which is

consistent with levels reported in other studies of stress in neonatal rats (Kalinichev et al., 2002; Spear et al., 1989; Sucheki et al., 1994; Kalinichev et al., 2002). Corticosterone levels have a diurnal pattern, with levels increasing throughout the progression of the light cycle (Ader, 1969; Dauchy et al., 2010). To account for these variations, corticosterone levels were correlated with time of day (specifically, minutes since lights were turned on for the day: 9 AM). Both the SC and SI pups showed significant correlations between time of day and corticosterone levels (SC: Pearson's $r = 0.5716$, $p < 0.01$; SI: Pearson's $r = 0.5582$, $p < 0.05$; Figure 6.6), while AE pups did not show a correlation (Pearson's $r = -0.1774$, p = n.s.), suggesting a disrupted diurnal corticosterone rhythm in the AE group.

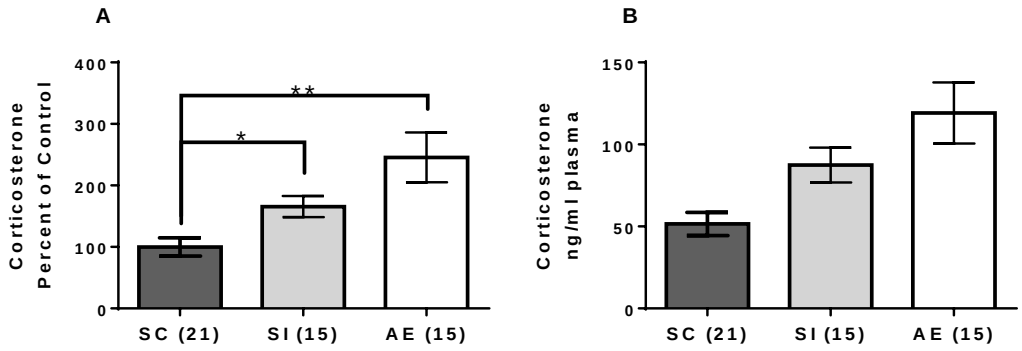

Figure 6.5. Plasma Corticosterone on PD10.

A) Both AE and SI animals showed an increase in corticosterone compared to the SC group. * p < 0.05; ** *p* < 0.01. SI and AE did not statistically differ from one another. Data is shown as mean percent of control ± SEM. B) Raw corticosterone levels for reference, displayed as ng/ml plasma ± SEM. AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control. Image reprinted with permission from Boschen et al., 2015.

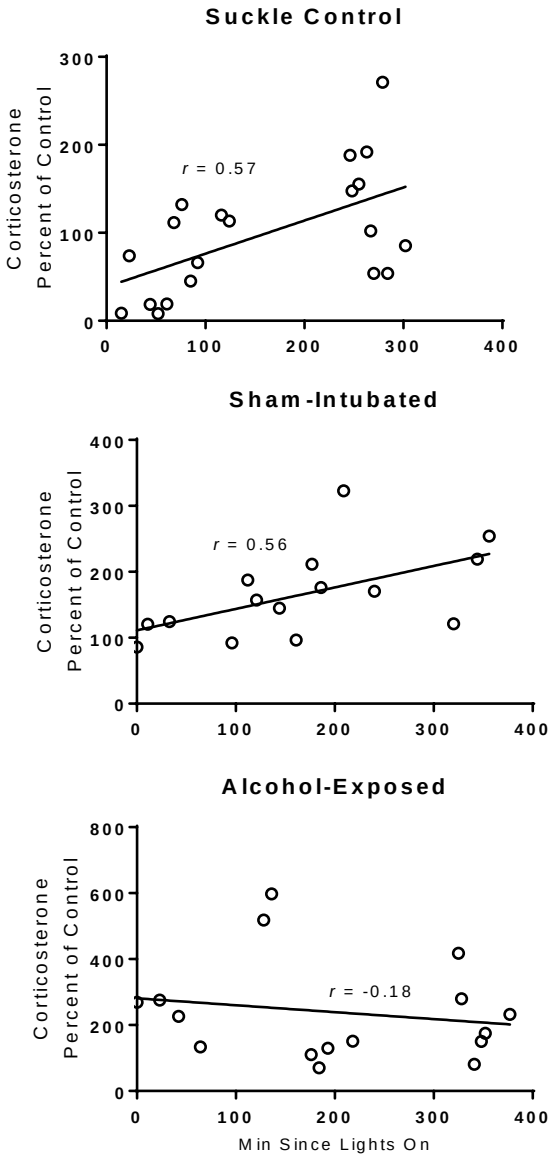

Figure 6.6. Plasma Corticosterone and Time of Sacrifice Correlations.

Significant correlations between time of day (minutes since lights on at 9 AM) and corticosterone levels were observed in SC and SI pups, but there was no correlation observed in AE animals. Pearson's *r* value given for each correlation. SC and SI: $p < 0.01$; AE: p = n.s. AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control. Image reprinted with permission from Boschen et al., 2015.

6.2.3 Discussion

Experiment 1 demonstrated that third trimester-equivalent binge-like alcohol exposure (AE) and sham intubation (SI) both influence expression of neurotrophic factors in the neonatal hippocampus. Specifically, both AE and SI groups showed: 1) more BDNF protein expression; 2) more exon IX (total) and IV mRNA; and 3) less methylation of DNA associated with exon I. Further, both the AE and SI groups showed higher levels of corticosterone; however, some measures were affected by alcohol exposure alone. TrkB receptor expression in the hippocampus was only increased in the AE animals compared to the SI pups, suggesting that the increase in TrkB receptors is specific to AE animals at this time point. Changes in exon I-driven gene expression were significant in AE pups compared to SC pups, suggesting a differential effect of alcohol exposure and sham intubation stress on exon-specific *Bdnf* gene transcription. *Bdnf* exon I DNA methylation was decreased in both AE and SI animals. To our knowledge, these results are the first to examine BDNF mRNA and protein levels, TrkB receptor levels, and *Bdnf* DNA methylation using the same model of alcohol exposure. Overall, these findings highlight the ability of developmental alcohol exposure or stress associated with method of delivery (repeated intubation) to alter BDNF and receptor levels in the neonatal hippocampus.

The findings of increased BDNF protein in the hippocampus following PD4-9 alcohol exposure are consistent with previous literature investigating the effects of developmental alcohol exposure on BDNF and other neurotrophins in various regions of the brain. Both pre- and postnatal alcohol exposure paradigms have been found to

increase hippocampal NGF (Angelucci et al., 1997; Heaton et al., 2000). Heaton and colleagues (2000) reported increased BDNF protein in the hippocampus on PD10 immediately following cessation of a week-long alcohol exposure paradigm. Interestingly, previous work with postnatal models has suggested a temporally-based fluctuation in cortical BDNF protein levels during the hours following a single alcohol exposure, with BDNF increasing immediately, decreasing after two hours, and then increasing again after 12 hours (Heaton et al., 2003). Temporally-specific alterations to BDNF levels following developmental alcohol exposure, could shed light on the functional significance of BDNF at each time point, particularly when taken together with levels of other pro-apoptotic and pro-survival molecules. More tissue collection time points are needed to fully address this question.

Expression of the *Bdnf* gene is regulated via transcription starting at one of at least nine promoter regions (Timmusk et al., 1993; Aid et al., 2007; Liu et al., 2007; Pruunsild et al., 2007). Consistent with the protein data (Figure 6.2A), the current study found that both postnatal alcohol exposure and sham intubation increased total *Bdnf* gene expression (Figure 6.3A). Interestingly, exon I-specific transcription was not increased to the same degree in SI pups as compared to AE animals (exon IV-specific expression was also not statistically significant from the SC group), suggesting that exon I did not contribute as much to the overall increases in *Bdnf* gene expression in the SI group compared to the AE condition. While both the AE and SI groups exhibit increased BDNF synthesis, the transcripts through which the protein is generated differs between the conditions. Both exons I and IV have been shown to

regulate experience-dependent BDNF release, and transcription can be differentially affected based on the type of stressor, brain region, and age of the animal (Metsis et al., 1993; Oliff et al., 1998; Nair et al., 2007; Suri et al., 2013). For example, Nair and colleagues (2007) reported that acute immobilization stress decreased expression of all *Bdnf* mRNA transcripts in the hippocampus while chronic immobilization stress resulted in both increased and decreased expression in an exon-specific pattern. Another study reported age-related alterations to *Bdnf* exon IV-driven gene expression following early life stress; specifically, mRNA levels were higher in stressed animals at 21 days and 2 weeks of age, but were lower than controls at 15 months. The impact of stress on the current results in discussed in more depth in Section 6.4.2.

Consistent with the increase in exon I-driven mRNA transcripts in the AE animals, DNA methylation was reduced in this group. Our observation of changes in methylation in AE pups is consistent with the handful of reports that have documented altered DNA methylation in other developmental alcohol models (Liu et al., 2009; Veazey et al., 2015; Govorko et al., 2012). Alcohol exposure during the third trimester-equivalent (postnatal day 2-10) was reported to cause global DNA hypermethylation in the hippocampus and prefrontal cortex on postnatal day 21 (Otero et al., 2012), though gene-specific effects were not assessed in this study. Finally, perinatal exposure (all three trimesters) increased the activity of DNA methyltransferases (Perkins et al., 2013). Interestingly, these results show that methylation at exon I was also decreased in the SI group without corresponding changes to gene expression. Other early life stressors have been shown to impact

methylation status of the *Bdnf* gene without concomitant changes in steady-state mRNA levels (reviewed in Blaze & Roth, 2015). Importantly, these paradigms demonstrate that alterations to methylation are highly dependent on the timing of tissue collection, the brain region analyzed, and the target gene in question. The fact that the SI group did not also show increased gene expression at exon I highlights the complicated interaction between DNA methylation and gene expression. Theoretically, it is possible that while SI animals exhibited more exon I DNA methylation other factors such as histone modifications or non-coding RNAs could be differentially affected in AE and SI animals, explaining a disconnect in methylation and transcription data.

BDNF and its specific receptor TrkB are critical for proper neural development and have distinct developmental timeline of expression, with increasing expression following the maturation of brain structures: the earlier maturing rodent brain regions (brainstem, midbrain) start to demonstrate the higher levels of expression during the first postnatal days (e.g., Maisonpierre et al., 1990, Fryer et al., 1996). BDNF mediates neuronal differentiation during development (Patapoutian & Reichard, 2001) and is required for the terminal differentiation of new neurons (Chan et al., 2008). Balanced expression of BDNF during critical periods in development is essential for synaptic maturation as BDNF potentiates excitatory and attenuates inhibitory synaptic transmission by acting on presynaptic terminals (Carvalho et al., 2008; Gao et al., 2014) and postsynaptic receptors (Tanaka et al., 1997; Brunig et al., 2001). BDNF may also play a vital role in mediating processes associating early life environment

with brain development and behavior (Branchi et al., 2006a,b, 2013). Thus, alterations to BDNF and TrkB signaling during development could result in long-lasting neuroplastic consequences. Furthermore, aberrant BDNF signaling could represent either a neuroprotective mechanism or a sign of dysregulation within the neurotrophic factor "push-pull" synchrony (McAllister et al., 1997).

The simultaneous increase in BDNF and TrkB protein in the hippocampus following postnatal alcohol exposure might represent a compensatory protective mechanism in which the brain responds to the damaging effects of alcohol through upregulation of growth factors. Elevated BDNF levels in hippocampus of sham-intubated animals could represent protective response to stress of intubation, however this increase was not accompanied by an upregulation in TrkB receptor. Increased release of neurotrophins have been reported following other types of brain trauma, including experimental models of traumatic brain injury and seizures (Dekosky et al., 1994; Hicks et al., 1997; Rudge et al., 1998). Postnatal alcohol exposure has also been shown to transiently increase BDNF protein levels in the hippocampus immediately following the last alcohol exposure (Heaton et al., 2000). The current results show that postnatal alcohol exposure also increases TrkB receptor expression and that upregulated levels of BDNF and TrkB persist for at least 24 following the last ethanol administration. As neurotrophins play a critical role in neuronal proliferation and survival and induce dendritic reorganization, upregulation of these proteins could potentially offset alcohol-induced neurotoxic and apoptotic effects. Some evidence supports a role for BDNF in neuroprotection (Heaton et al., 1994; Mitchell et al.,

1999; Nagahara & Tuszynski, 2011), though exogenously administered BDNF does not necessarily result in therapeutic effects (Rudge et al., 1998; Nagahara & Tuszynski, 2011). However, any deviation from normal levels of BDNF could result in detrimental effects, as many neurotrophic factors and other growth hormones act in synchrony and levels are precisely developmentally-timed. Prolonged overexpression of neurotrophins, such as BDNF, could result in aberrant synaptic connectivity (Miller & Al-Rabiai, 1994), dendritic arborization, or even increased apoptosis via activation of the p75 receptor (Casaccia-Bonnefil et al., 1996). While not systematically studied in the current experiment, the potential role of withdrawal effects should also be taken into consideration with the current results, given that animals were sacrificed 24 hours following the last alcohol exposure and physiological signs and symptoms of withdrawal start as soon as six hours following alcohol in adult animals (Becker, 2000). Alcohol withdrawal effects are thought to be in part due to neuronal excitotoxicity following a compensatory upregulation of NMDA receptors following exposure (Young et al., 2010, Idrus et al., 2014), which might contribute “rebound hyperexcitability” in the hippocampus of human alcoholics (described in Becker, 2000). CNS hyperexcitability could play a role in the observed increase in BDNF and TrkB in AE pups in the current study.

6.3 Experiment 2: Impact of Neonatal Alcohol Exposure on Number and Activation State of Microglia in the Developing Rat Hippocampus[5]

Recent work has suggested alcohol-induced neuroinflammation, as measured by increased activation of microglia and levels of associated cytokines, as another source of damage in various models of alcohol exposure, both during development and in adulthood (McClain et al., 2011; Saito et al., 2010; Kane et al., 2011; Tiwari & Chopra, 2011; Marshall et al., 2013; Drew et al., 2015; Topper et al., 2015). Alcohol exposure during the third trimester-equivalent induces waves of apoptosis in the hippocampus, possibly triggering microglial migration to the damaged tissue and activation of the resident microglia to phagocytose dying cells and debris (Miller, 1998; Ikonomidou et al., 2000; Smith et al., 2015). Microglial activation in response to alcohol has been suggested to not only be a consequence of the insult also but a source of inflammation and tissue damage (Marshall et al., 2013).

The rodent immune system begins to develop during gestation and continues through the first two weeks of neonatal life, with microglia beginning colonization on embryonic days 9-10 in a brain-region specific manner (Chan et al., 2007; Ginhoux et al., 2010). Microglia respond to immune challenges through release of pro- and anti-inflammatory cytokines and phagocytosis of dying neurons and pathogens. Aberrant microglia activation during development can lead to chronically increased levels of pro-inflammatory cytokines which could lead to neurodevelopmental and

[5] Experiment 2 text and figures reproduced with permission from Boschen et al., 2016.

psychopathological disorders (Cai et al., 2000; Meyer et al., 2006; Urakubo et al., 2001) or exaggerated immune responses to challenges later in life (Bilbo & Schwarz, 2009; 2012).

Microglia can express both pro- and anti-inflammatory cytokines in response to an immune challenge. The release of cytokine and phagocytosis of debris is linked with microglial morphology (Gehrmann et al., 1995; Neumann et al., 2009). Microglia exhibit various activation states characterized by changes to their physical shape and size. Resting or quiescent microglia are characterized by a small soma and long, thin processes for surveying the local microenvironment for pathogens or injury (Figure 6.7A). Once a pathogen has been detected the microglia's soma enlarges and its processes shorten and thicken. A fully activated microglia displays a round, amoeboid shape with either very short or complete lack of processes (Figure 6.7B; Nimmerjahn et al., 2005; Olah et al., 2011; Fu et al., 2014). Pro-inflammatory cytokines, released by activated microglia and macrophages, have cytotoxic effects and can induce further cell loss and tissue damage, while anti-inflammatory cytokines inhibit expression of pro-inflammatory cytokines, initiate cellular repair, and are generally thought to be neuroprotective. A balance between the actions of these cytokines dictates how the brain recovers from immune challenges.

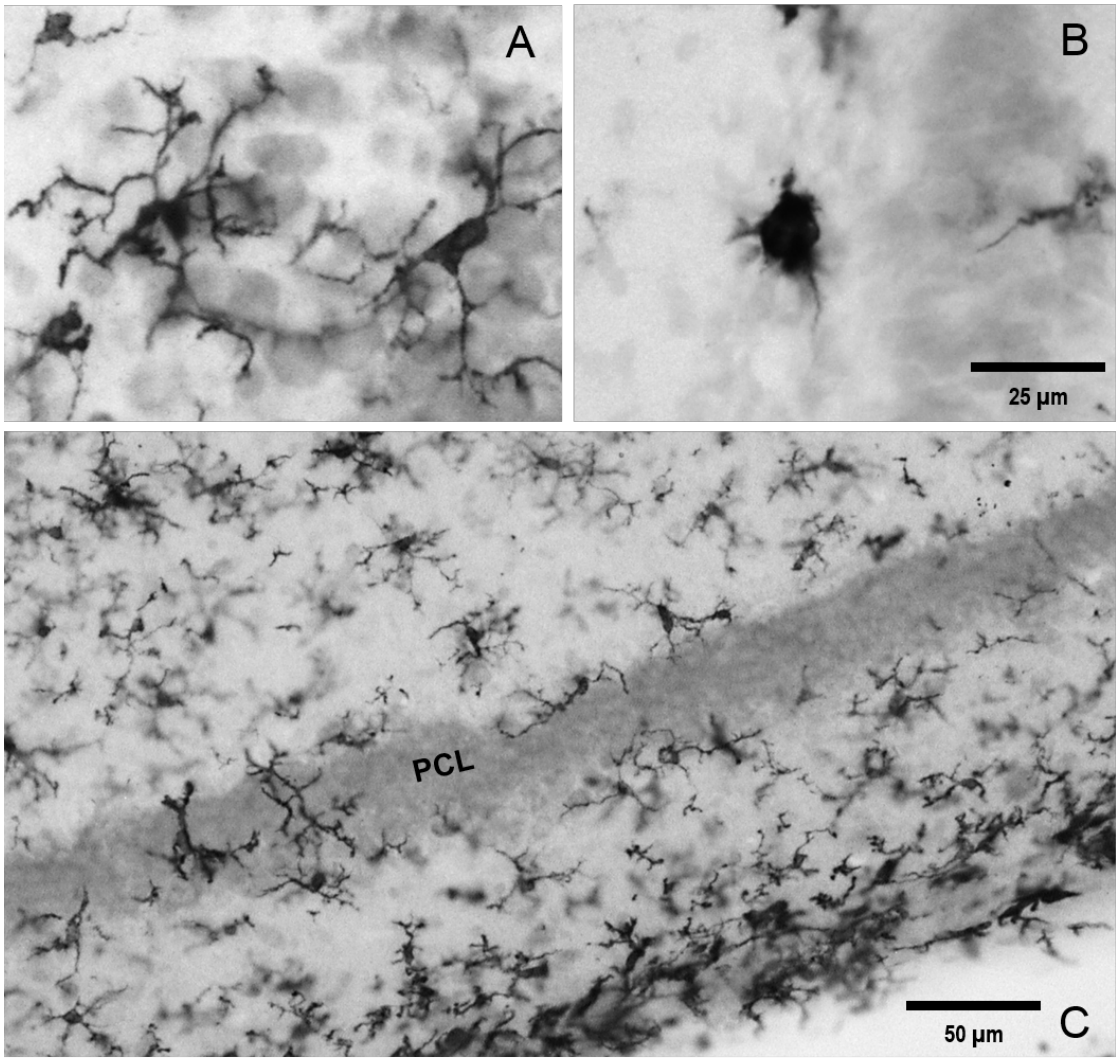

Figure 6.7. Representative Images of Iba-1+ Microglia in the PD10 Rat Hippocampus.

A) Quiescent microglia with small somas and long, thin processes. B) Fully activated amoeboid microglia with a large, round soma and very short processes. Both images taken with a 40x lens. C) Iba-1 immunostaining in the neonatal rat hippocampal CA1. Image taken with a 20x lens. Image reprinted with permission from Boschen et al., 2016.

The current study investigates whether third trimester-equivalent (postnatal days [PD]) 4-9) binge-like alcohol exposure affects microglial activation in the neonatal rat hippocampus through analysis of subregion-specific microglial number and territory (area) occupied by microglial body and processes. Cell territory provides an indirect measure of activation state, as a smaller area would indicate a more activated morphology (Drew & Kane, 2014). Neuroinflammation was also measured through analysis of gene expression of four pro-inflammatory cytokines: IL-1β, TNF-α, CD11b, and CCL4, and one anti-inflammatory cytokine: TGF-β. We hypothesized that our model of alcohol exposure would increase the number of microglial cells in hippocampus of neonatal rats, activate existing microglia, and increase production of pro-inflammatory cytokines. This study will give a starting point for assessing whether aberrant neuroimmune activation via neonatal alcohol exposure could have long-term consequences for the brain and behavior.

6.3.1 Materials and Methods

6.3.1.1 Subjects

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 h 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) and markings for pup identification were made by injecting a small volume of non-toxic India black ink into the paws. On PD 4, a split-litter design was used to assign the pups to one of three experimental groups: suckle control (SC), sham-

intubated (SI) or alcohol-exposed (AE). AE and SI pups were represented in the same litter and SC pups from a separate litter to allow these pups to be left completely undisturbed aside from daily weighing. Following the alcohol exposure, pups were left undisturbed with the dam until sacrifice on PD10. A total of 60 male pups from 21 litters were used for the data presented here. More specifically, for stereology, 10 AE, 9 SI and 8 SC pups were used; for gene expression, 9 AE, 12 SI and 12 SC pups were used. Animals used for stereology were also administered a 50 mg/kg bromodeoxyuridine (BrdU) i.p. injection 2 hours prior to sacrifice to be used to answer additional questions not reported here. All procedures were carried out in accordance with the animal use protocol approved by University of Delaware Institutional Animal Care and Use Committee and in accordance with NIH's Animal Care Guidelines.

6.3.1.2 Alcohol Exposure and BAC analysis

On PD 4-9, AE pups received 5.25 g/kg/day alcohol in a milk formula (11.9% v/v) in 2 doses, 2 hours apart via intragastric intubation (Figure 6.1A). On PD4, AE pups also received two milk-only feedings as a caloric supplement 2 and 4 hours following the second alcohol dose; on PD 5-9, AE pups were given one milk-only feeding two hours following the second alcohol dose. SI pups were intubated alongside the AE rats as an intubation control but received no liquid solution. Previous work has suggested that delivery of milk formula to the SI pups could result in accelerated weight gain. SC pups remained undisturbed with the dam except for daily weighing to insure proper development.

On PD 4, 90 minutes following the second alcohol exposure, blood samples were collected through tail clippings from both the AE and SI groups. Blood samples from the AE group were centrifuged (15,000 rpm/25 minutes) and the plasma collected and stored at -20ºC until analysis. BACs were analyzed using an Analox GL5 Alcohol Analyzer (Analox Instruments, Boston, MA).

6.3.1.3 Immunohistochemistry

On PD10, animals were deeply anesthetized (ketamine/xylazine cocktail), transcardially perfused (0.1M PBS with heparin followed by 4% paraformaldehyde) and the brains were stored in 4% paraformaldehyde for 24 hours. The brains were then transferred into 30% sucrose in 4% paraformaldehyde until sectioning. Brains were sectioned coronally at 40 µm through the entirety of the hippocampus. Microglia were identified with microglia-specific marker ionized calcium-binding adaptor molecule 1 (Iba-1). Two control sections per animal were processed identically to the immunolabeled sections but were placed in blocking solution only instead of primary antibody during the steps listed above; staining of these sections insured antigen-specific labeling. The following protocol was used:

1. Wash tissue in 0.1M Tris-buffered saline (TBS) 3 x 5 min
2. Incubation in 0.6% H2O2 in 70% methanol for 10 min
3. Incubation in blocking solution (3% normal goat serum + 0.1% Triton-X100 made in 0.1M TBS) for one hour at room temperature

4. Incubation of sections in primary antibody (rabbit anti-Iba-1, 1:5000 dilution, Wako Chemicals) in washing solution (3% normal goat serum in 0.1M TBS) for 24 hours at 4°C
5. Wash tissue in 0.1M TBS for 3 x 5 min
6. Incubate sections in secondary antibody (goat anti-rabbit biotinylated secondary, dilution 1:200, Vector) in washing solution for one hour at room temperature
7. Wash tissue in 0.1M TBS 2 x 10 min
8. Wash tissue in washing solution 1 x 10 min
9. Incubate tissue in Avidin-Biotin Complex solution (Vector) mixed in washing solution for one hour at room temperature
10. Wash tissue in 0.1M TBS 3 x 5 min
11. Visualize immunolabeling with 0.5 mg/ml nickel-enhanced diaminobenzedine (DAB) and 0.33 µl/ml H_2O_2 in 0.1M TBS
12. 0.1M TBS rinse for 1-2 sec
13. Wash tissue in 0.1M TBS 2 x 5 min

6.3.1.4 Cell Quantification and Cell Territory

Sections used for cell quantification were selected in a systematic random manner ($1/12^{th}$ sampling fraction). Iba-1+ cells were counted using unbiased stereology within a known volume of CA1, CA3, and the dentate gyrus subregions of the hippocampus (CA1 shown in Figure 6.7C), using the optical fractionator probe (Stereo Investigator, MBF Bioscience, Williston, VT). All regions of each

hippocampal subfield were included in the analyses (e.g. for dentate gyrus, the molecular layer, granule cell layer, and the hilus were counted). The sampling grid was set to 200 x 200 µm and the counting frame set to 100 x 100 µm. A dissector height of 16 µm was used and a guard zone of 2 µm on either side of the section was used. For all counts, the mean coefficient of error (CE) did not exceed the recommended 0.1 (Gunderson, 1986).

As an indirect estimate of microglia morphology and activation state, total cell area or "territory" (Drew et al., 2015) was measured for the cell layers of CA1, CA3, and dentate gyrus using NeuroLucida software (v. 10.52, MBF BioScience, Williston, VT). For each section, the area encompassed by an individual microglia was measured by tracing a contour from the tip of each microglial process to the next. Five pseudorandomly selected microglia were traced per section from microglia located within the cell layer or immediately adjacent with at least one process extending into the cell layer. For each animal, 5-8 sections were used per subregion, as the regions were not always present in the most dorsal or ventral sections. The areas of the microglia were the averaged within and across sections. Activated microglia are usually characterized by shorter, thicker processes and a larger soma (Figure 6.7A) whereas resting microglia have longer, thinner processes (Figure 6.7B), meaning that smaller microglia cell territory would indicate a more activated morphology.

6.3.1.5 Gene Expression Analysis

On PD10, animals were rapidly decapitated and the brains frozen with -20ºC 2-methylbutane and stored at -80°C until processing. Dorsal and ventral hippocampus

were dissected on dry ice and DNA/RNA were extracted (Qiagen, Valencia, CA). Quantification and analysis of nucleic acid quality were performed with spectrophotometry (NanoDrop 2000, ThermoScientific) and reverse transcription was performed using the Quantitect Reverse Transcription Kit (Qiagen). cDNA was amplified with real-time PCR (Bio-Rad, CFX96). Gene expression for *TNF*, *ITGAM* (CD11b), *CCL4*, *IL10,* and *TGFB1* was assessed using Taqman probes (Life Technologies), and *tubulin* as a reference gene (Table 2). *IL1B* gene expression was assessed using SYBR (Bio-Rad) and the appropriate forward and reverse primers, with *GAPDH* used as a reference gene. The sequences of primers for *IL1B* and *GAPDH* were: *IL1B* forward: GAAGTCAAGACCAAAGTGG, reverse: TGAAGTCAACTATGTCCCG; GAPDH forward: GTTTGTGATGGGTGTGAACC, reverse: TCTTCTGAGTGGCAGTGATG (Posillico & Schwarz, 2015). Levels of *tubulin* and *GAPDH* were not altered by neonatal treatment, as determined by one-way analysis of variance ($F < 1$). All reactions for each gene target and reference were run in triplicate, except for *IL1B* and *GAPDH* which were run in duplicate. Product specificity was verified by agarose gel electrophoresis or melt curve (*IL1B* and *GAPDH*). Levels of *IL10* were not detectable in the PD10 brain and thus will not be discussed in the Results section (see Discussion for further information).

Gene	Encoded Protein	Catalog No.
TNF	Tumor necrosis factor-α (TNF-α)	Rn01525859
ITGAM	Cluster of differentiation molecule 11B (CD11b)	Rn00709342
CCL4	Chemokine (C-C motif) ligand 4 (CCL4)	Rn00671924
IL1B	Interleukin-1β (IL-1β)	Custom primers
TGFB1	Transforming growth factor-β (TGF-β)	Rn00572010
Tubulin	Tubulin	Rn01435337
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Custom primers

Table 6.2. List of Gene Expression Primers.

Table reprinted with permission from Boschen et al., 2016.

6.3.1.6 Statistical Analyses

Animal weights for PD4, 9, and 10 were averaged within neonatal condition and analyzed using a repeated-measures analysis of variance (ANOVA; day x neonatal condition) followed by *post hoc* tests when appropriate. For BACs, average BAC for each animal (2-3 analyses per animal) ± standard error of the mean (SEM) are reported as mg/dl. Microglia number and cell territory were assessed using one-way ANOVA. *Post hoc* tests (Tukey's) were used when appropriate. The comparative Ct method was used to obtain the relative fold change in gene expression of experimental (AE or SI) vs. the average of controls (SC) per plate (Livak and Schmittgen, 2001). Comparisons between the AE and SI groups were performed using unpaired *t*-tests and comparisons between the experimental (AE and SI) and the control group (SC) were analyzed with a one-sample *t*-test (hypothetical value set to 1.0) as commonly done in the field (e.g. Roth et al., 2009; Blaze & Roth, 2013; Debruin et al., 2014).

Using this method, a mean value of 1 indicates no change in mRNA transcript level compared to the SC group. Differences were considered to be statistically significant at $p < 0.05$ and nonsignificant trends at $p < 0.1$ are also reported. Outliers for each neonatal treatment group were identified using Grubb's test. No outliers were removed for cell count or cell territory; for gene expression data, the number of outliers removed for each gene assay was as follows: *CCL4*: 3, *ITGAM* (CDllb): 2, *TGFB1*: 2, *IL1B*: 1, *TNF*: 0. Weights analyses were run using SPSS 16 and all other statistical analyses were run using Prism 6 software (GraphPad Inc.).

6.3.2 Results

6.3.2.1 Body Weights and BACs

Animals were weighted daily during the alcohol exposure paradigm. PD4, 9 and 10 weights were compared to assess potential changes in nutritional status due to neonatal condition. A repeated-measures ANOVA revealed a significant main effect of day ($F_{(2,114)} = 2278.657$, $p < 0.001$), as all animals increased in body weight across the treatment period. A significant day x neonatal condition interaction was found ($F_{(4,114)} = 14.887$, $p < 0.001$). One-way ANOVAs were then ran separately for PD4, 9 and 10. For PD4, pups weighed similarly regardless of neonatal condition ($F_{(2,57)} = .413$, $p = 0.664$), however on PD9 and 10, a significant main effect of neonatal condition was found ($F_{(2,57)} = 7.764$, $p = 0.001$; $F_{(2,57)} = 7.243$, $p = 0.002$). Post hoc Tukey's tests revealed that on PD9 and 10, AE animals weighed significantly less than SI and SC pups (PD9: AE vs. SI: $p = 0.001$, AE vs. SC: $p = 0.024$, SI vs. SC: $p = 0.499$; PD10: AE vs. SI: $p = 0.001$, AE vs. SC: $p = 0.031$, SI vs. SC: $p = 0.519$).

Previous work from our lab has shown that this difference in weight gain is limited to the neonatal treatment period, as AE animals are indistinguishable from control animals by PD30 (Hamilton et al., 2012; Boschen et al., 2014). However, the role of malnutrition in the AE pups must be considered.

BACs were collected from a tail clip on PD4, 90 min following the second alcohol dose. One AE animal was excluded due to too little plasma being collected to accurately perform the Analox analysis. The average BAC was 358.97 mg/dl (± 88.14 SEM), which is consistent with other published BAC levels from our lab using the same alcohol exposure model (Hamilton et al., 2012; Boschen et al., 2014).

6.3.2.2 Microglial Cell Number

Total microglial cell counts were estimated using unbiased stereological analysis of Iba-1+ cells in hippocampal CA1, CA3, and dentate gyrus subregions. Subregion-specific effects were observed, with significant decreases in microglial number found in AE and SI compared to SC in CA1 and dentate gyrus, but not in CA3 (Figure 6.8A-C). Specifically, in CA1, a significant main effect of neonatal treatment was also found ($F_{(2,18)} = 5.433$, $p = 0.0143$), with significantly decreased cell numbers found for the AE and SI groups compared to SC ($p < 0.05$, Tukey's *post hoc*; Figure 7.8A). In the dentate gyrus, there was a main effect of neonatal condition ($F_{(2,18)} = 25.56$, $p = 0.0001$), with Tukey's *post hoc* analysis finding a significant different between both AE and SI groups and the SC group ($p < 0.05$; Figure 6.8C). Notably, while animals from both AE and SI postnatal conditions had a lower numbers of microglia than SC, a significant higher number of Iba-1+ cells was found in the AE

animals compared to the SI ($p = 0.005$). There were no significant effects in CA3 ($p > 0.05$, Figure 6.8B).

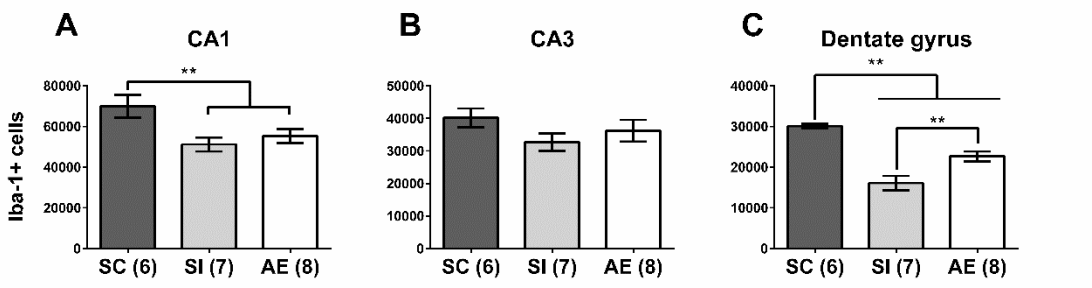

Figure 6.8. Microglial Cell Counts in the PD10 Hippocampus.

Number of Iba-1+ microglia were significantly decreased in hippocampal CA1 (A) of AE and SI animals compared to the SC group. No effect of neonatal treatment was found in CA3 (B). In dentate gyrus (C), AE and SI animals had fewer microglia compared to the SC group, but the AE group also had significantly more microglia compared to the SI animals. AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control. ** = $p < 0.01$. Image reprinted with permission from Boschen et al., 2016.

6.3.2.3 Microglial Cell Territory

Area of cell territory was measured in microglia residing within the cell layer of the CA1, CA3, and dentate gyrus subregions. Significant decreases in microglial cell territory (measured in μm^2) were found in alcohol-exposed animals in CA1 and dentate gyrus (Figure 6.9A-C). For CA1, a main effect of neonatal condition was found ($F_{(2,15)} = 15.14$, $p = 0.0003$), with Tukey's post hoc analysis indicating a significant decreased in cell territory in the AE group compared to both the SI and SC groups ($p < 0.05$ and 0.001, respectively; Figure 6.9A). SC and SI did not differ in cell territory from one another. In CA3, a main effect of neonatal condition was found

($F_{(2,15)} = 7.779$, $p = 0.0048$), showing a significant decrease in cell territory for AE animals compared to both the SI and SC groups ($p < 0.01$ and $p < 0.05$, respectively; Figure 6.9B). Again, SI and SC groups did not differ. For dentate gyrus, there was a main effect of neonatal condition ($F_{(2,15)} = 4.487$, $p = 0.029$), with a significant decrease in cell territory in AE animals compared to SC, but not SI, animals ($p < 0.05$, Figure 6.9C). SI did not differ significantly from the SC or AE groups.

Microglia were then classified into size categories to better visualize the differences in cell territory between AE and control animals (Figure 6.9D-G). The microglia were classified as "small" (<1000 μm^2), "medium" (1000-2000 μm^2), or "large" (>2000 μm^2) (representative illustrations shown in Figure 6.9) and the percentage of each category was calculated from the total number of cells analyzed for each animal. Most microglia in all regions and neonatal conditions fell into the "medium" classification, but some shifts in microglial size were observable between conditions. For all three regions, one-way ANOVAs were run to analyze shifts in the number of microglia classified as "small" or "large" in each area, as these categories would capture the most activated ("small") or least activated ("large") phenotypes. In CA1, a significant main effect of neonatal condition was found for number of "small microglia" ($F(2,15) = 5.266$, $p = 0.019$, with *post hoc* tests (Tukey's) revealing a significant differences between AE and SI ($p < 0.05$) and AE and SC ($p < 0.05$), but not SI and SC. For "large" microglia in CA1, a main effect of neonatal treatment was found ($F(2,15) = 8.661$, $p = 0.003$), with significant differences between AE and SC ($p < 0.01$) and SC and SI ($p < 0.05$), but not AE and SI. In CA3, a significant effect of

neonatal treatment was revealed for “small” microglia ($F(2,15) = 3.824$, $p = 0.0455$), with differences between AE and SI ($p < 0.05$), but not between SC and SI or SC and AE. For “large” microglia in CA3, a significant main effect of treatment was found ($F(2,15) = 6.602$, $p = 0.009$), with differences between AE and SI ($p < 0.05$) and AE and SC ($p < 0.05$), but not between SI and SC. In dentate gyrus, there was no main effect of neonatal condition on the percentage of “small” or “large” microglia ($p > 0.05$), suggesting that the shifts in microglial size in the dentate gyrus, while sufficient to be statistically significant between SC and AE when assessing average cell territory, were more subtle then the shifts found in the other two brain regions.

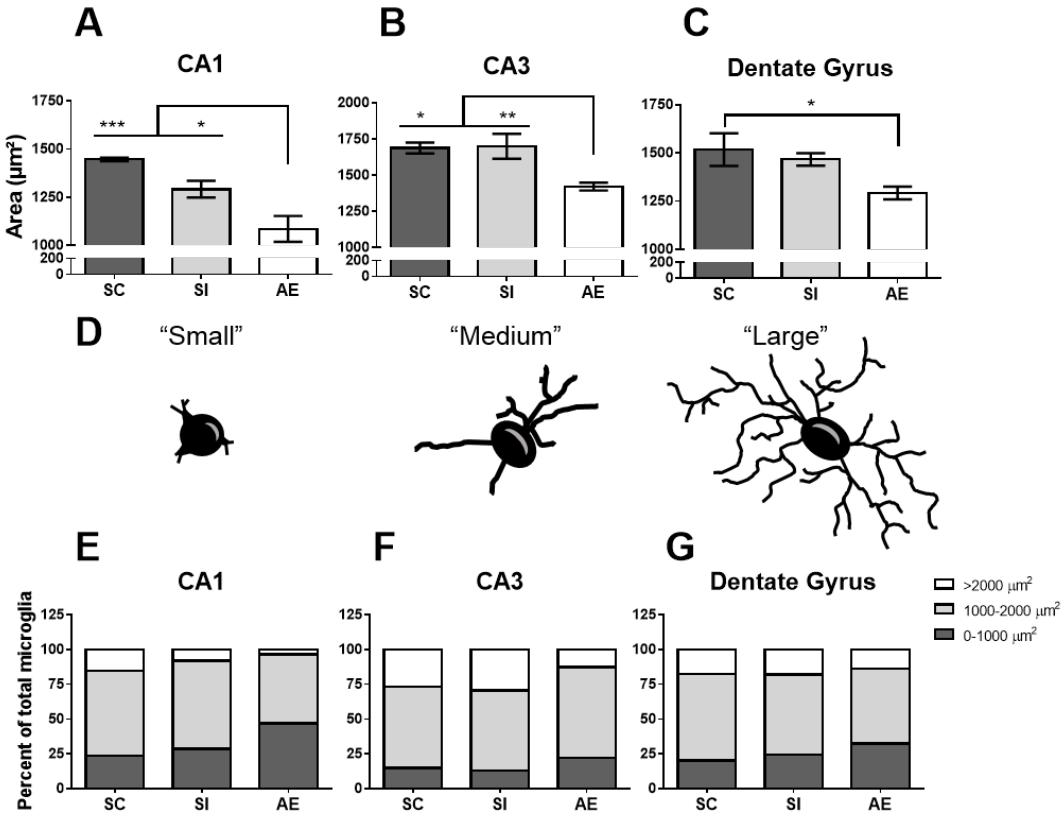

Figure 6.9. Microglia Cell Territory in the PD10 Hippocampus.

A) In CA1, the average area covered by each microglia was decreased in the AE group compared to the SI and SC groups ($p < 0.05$ and 0.001, respectively). B) In CA3, cell territory was decreased in the AE group compared to SI and SC ($p < 0.05$ and 0.01, respectively). C) The AE group had significantly smaller microglia in the dentate gyrus (DG) compared to the SC group ($p < 0.05$), but was not significantly different from SI. Visualization of the size of the microglia measured in each region when categorized as small, medium or large. D) Representative drawings of small, medium, and large microglia. E) CA1, F) CA3, G) DG. AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Image reprinted with permission from Boschen et al., 2016.

6.3.2.4 Pro- and Anti-Inflammatory Cytokine Gene Expression

Cytokine gene expression was assessed using whole hippocampal tissue. We found that levels of three of the pro-inflammatory cytokines (IL-1β, TNF-α, and CD11b) were increased in both AE and SI groups compared to the SC group and unpaired *t*-tests revealed no significant difference between AE and SI ($t < 1$, $p > 0.05$) (Figure 6.10A-C). For IL-1β, both AE and SI groups had increased levels compared to SC (for AE vs. SC: $t(8) = 3.160$, $p = 0.013$; for SI vs. SC: $t(7) = 3.778$, $p = 0.007$). For TNF-α, both AE and SI were increased compared to SC (for AE vs. SC: $t(7) = 3.096$, $p = 0.017$; for SI vs. SC: $t(9) = 3.34$, $p = 0.009$). For CD11b, levels were increased in both AE and SI groups compared to SC (for AE vs. SC: $t(8) = 2.73$, $p = 0.026$; for SI vs. SC: $t(11) = 3.275$, $p = 0.007$). Interestingly, while pro-inflammatory cytokine CCL4 was significantly increased in both AE and SI compared to the SC group (for AE vs. SC: $t(5) = 4.821$, $p = 0.0048$; for SI vs. SC: $t(8) = 3.716$, $p = 0.0059$), an unpaired *t*-test revealed that levels of CCL4 were significantly increased in AE compared to SI animals as well ($t(13) = 2.559$, $p = 0.0238$; Figure 6.10D). Levels of anti-inflammatory cytokine TGF-β were increased in AE animals vs. SC and SI controls (AE vs. SC: $t(8) = 2.927$, $p = 0.019$; AE vs. SI: $t(17) = 2.439$, $p < 0.05$; Figure 6.10E). These results indicate that while the SI group also show increases in some pro-inflammatory cytokine activity was increased in both AE and SI groups in comparison with SC, neonatal AE causes even greater expression of CCL4 and induces an anti-inflammatory response on PD10.

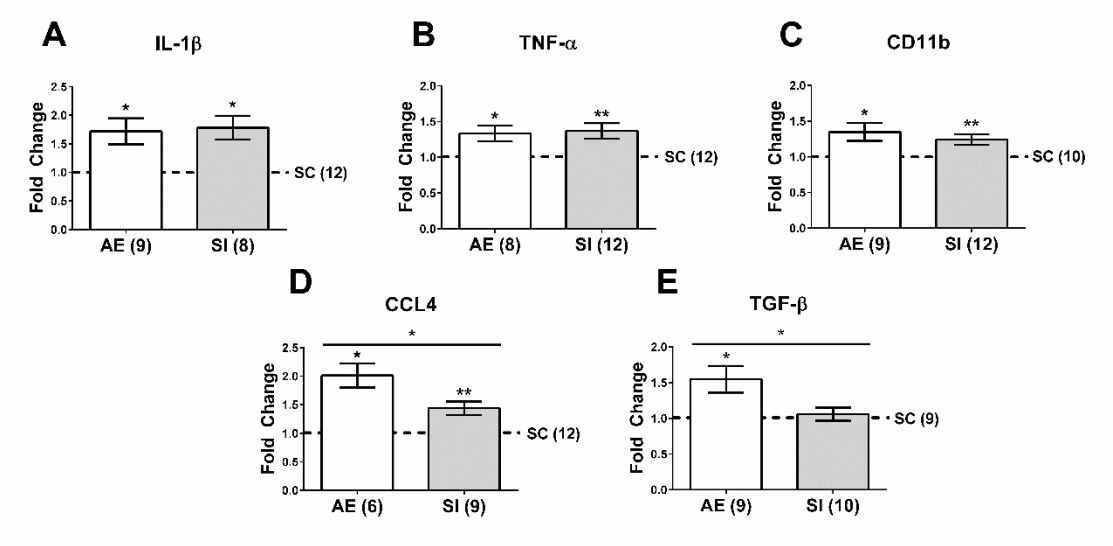

Figure 6.10. Pro- and Anti-Inflammatory Gene Expression in the Neonatal Hippocampus.

Both AE and SI groups had elevated levels of IL-1β (A), TNF-α (B), and CD11b (C) compared to SC. For CCL4 (D), both AE and SI groups showed increased gene expression, however levels of CCL4 in the AE group were significantly higher than in the SI group ($p < 0.05$). E) Gene expression of anti-inflammatory cytokine TGF-β were significantly increased in the AE group over the SI and SC animals ($p < 0.05$). Data is expressed as a fold change from the suckle control (SC) group (shown as 1 on graphs). AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control. * = $p < 0.05$, ** = $p < 0.01$. Image reprinted with permission from Boschen et al., 2016.

6.3.3 Discussion

Experiment 2 investigated whether third trimester-equivalent binge-like alcohol exposure (PD 4-9) induces microglial response in the neonatal hippocampus. Overall, we found two main factors contributing to increases in neuroinflammation in the neonatal brain: alcohol exposure and stress of handling/intubation. We found both common and unique effects on microglial cell number and activation state. Specifically, PD4-9 alcohol exposure (AE) uniquely decreased microglia cell territory

in the dentate gyrus, CA3 and CA1, indicative of microglia with an activated morphology, and increased CCL4 and TGF-β gene expression compared to both control groups on PD10. We also found fewer microglia in the dentate gyrus and CA1 subregions of AE and sham-intubated (SI) animals compared to suckle controls (SC), and that these groups showed similarly increased levels of pro-inflammatory cytokines IL-1β, TNF-α, and CD11b. Decreased microglial number and enhanced expression of pro-inflammatory molecules in the AE and SI groups likely represent the brain's generalized immune response to insult. Importantly, neuroinflammation was enhanced in AE animals as measured by increased microglial activation, increased expression of CCL4, and upregulation of the anti-inflammatory response as measured by TGF-β.

Region-specific decreases in Iba-1+ microglia numbers in the AE and SI groups compared to the SC group were seen in the dentate gyrus and CA1 regions, but not CA3. These results differ from the previously demonstrated increased proliferation of microglia in response to high alcohol doses in adolescence and adulthood (Ward et al., 2009; McClain et al., 2011; Marshall et al., 2013). However, ethanol has been reported to be toxic to cultured microglia and caused loss of cerebellar microglia *in vivo* (24 hours following PD3-5 3.5 mg/kg ethanol in the neonatal mouse), with remaining microglia expressing activated morphological characteristics (Kane et al., 2011). Additionally, the stress hormone corticosterone can act on microglial glucocorticoid receptors to inhibit microglial proliferation in culture (Ganter et al., 1992), which could be playing a role in the current study, as our model of alcohol exposure elevates levels of plasma corticosterone in AE and SI animals (Experiment

1). The current study cannot discern whether the observed decrease in microglia number was due to decreased proliferation, increased apoptosis of the microglia themselves, or changes to microglial migration. It is possible that the number of microglia across the entire brain remained constant across conditions and the decreases in the hippocampus were due to the microglia being recruited to other brain areas in the SI and AE groups, as amoeboid microglia have been reported to be highly motile (Brockhaus et al., 1996; Stence et al., 2001). Interestingly, a small but significant increase in microglial number was found in the AE group compared to SI in the dentate gyrus, suggesting that alcohol exposure and stress intubation alone could affect microglial number at differing rates or on a different time course following the first insult. Our lab is currently exploring a more in-depth time course of the neuroimmune response following alcohol exposure to assess how these changes present across days.

Area of cell territory was used in this study as an indirect measure of microglial morphology and activation, as activated microglia have shorter processes with larger cell bodies compared to the long, thin processes found on resting microglia. The current study found that in CA1, CA3, and dentate gyrus cell layers, the microglia in AE animals covered less territory with their processes, indicating that the microglia are activated following the alcohol exposure. While AE and SI animals both have decreased numbers of microglia in CA1 and dentate gyrus, neonatal alcohol exposure causes the remaining microglia to exist in a more activated state. A similar method has been used previously to show that neonatal alcohol exposure reduces cell

territory in the developing mouse hippocampal CA1, cerebellum and parietal cortex (Drew et al., 2015). The current study extends these results to a commonly used rat model of FASD, and shows increased microglial activation in all three subregions of the hippocampus. Further work is being done to elucidate differences in the time course of microglial activation and cytokine release in AE and SI pups. It is important to consider the potential effect of alcohol withdrawal on microglial activation in the current study, since the physiological signs and symptoms of withdrawal can begin in as little as 6 hours following alcohol administration in adult animals (Becker, 2000) and the pups in the current study were sacrificed 24 hours following the last alcohol exposure. Withdrawal symptoms are linked to neuronal excitotoxicity following a compensatory upregulation of NMDA signaling (Young et al., 2010, Idrus et al., 2014) and multiple withdrawal episodes are thought to act via elevated extracellular glutamate to prime microglia towards a more inflammatory response to subsequent alcohol exposures (Nixon et al., 2008; Ward et al., 2009; McClain et al., 2011), in part explaining why the microglia in the AE group had a more activated phenotype (smaller cell territory) compared to the SI and SC groups.

Alcohol can alter cytokine levels in a variety of tissues other than the brain, such as plasma, lungs, and liver (Crews et al., 2006). Both pro- and anti-inflammatory cytokines are found in both the peripheral and central nervous systems and can be produced by activated macrophages (including microglia), as well as certain other cell types, including astrocytes (Choi et al., 2014), other immune cells such as leukocytes and lymphocytes, dendritic cells, and, in the case of TNF-α, neurons. The current

study found a significant increase in gene expression of the pro-inflammatory cytokines IL-1β, CD11b, TNF-α, and CCL4 in both the AE and SI groups compared to the SC group, suggesting that while there were fewer microglia colonizing the hippocampus in both groups, the remaining cells are releasing increased levels of pro-inflammatory cytokines. Since there is evidence that astrocytes can also release cytokines (Choi et al., 2014) in response to alcohol *in vitro* (Blanco et al., 2005), the role of cytokine production in these cells should be investigated as an alternate source of neuroinflammatory signaling.

The influence of some cytokines, such as CCL4, seems to be limited to the acute, local inflammatory response, while overexpression of other cytokines, such as IL-1β or TNF-α, can activate pro-apoptotic pathways (Denes et al., 2012; Hogquist et al., 1991; Bilbo & Schwarz, 2009; 2012). Cytokines can have profound effects on cell survival and neuroplasticity, as microglia play an important role in synapse maintenance. In particular, IL-1β is known to be a critical mediator of inflammation involved in cellular processes such as proliferation and apoptosis, with recent work demonstrating an important role for IL-1β in learning and memory (Bilbo & Schwarz, 2009; 2012). Furthermore, IL-1 can induce rapid suppression of the peripheral immune system (Weiss et al., 1989). Aberrant activation of IL-1β or other cytokines during development could contribute to both behavioral deficits and altered immune function in adulthood. Most of the recent neonatal literature, including current study, reports cytokine mRNA levels rather than levels of secreted protein (Drew et al., 2015; Topper et al., 2015). Research suggests that the correlation between cytokine mRNA

and protein levels is not always strong and is dependent on the tissue type and cytokine analyzed (Zheng et al., 1991; Hirth et al., 2001). Most analysis of cytokines in the adult rodent brain have assessed protein levels (Marshall et al., 2013; McClain et al., 2011; Tiwari & Chopra, 2011), possibly due to the tissue requirement for successful protein analysis being greater than that needed for RT-PCR. However, future work could use enzyme-linked immunosorbent assay (ELISA), which is more sensitive than other protein measures, to correlate protein and mRNA levels of cytokines in the neonatal brain.

Our model of alcohol exposure has been shown to impair long-term neuroplastic and behavioral trajectories related to learning and memory (Johnson & Goodlett, 2002; Klintsova et al., 2007; Hunt et al., 2009; Murawski et al., 2012; Thomas & Tran, 2012; Hamilton et al., 2010, 2011, 2012, 2014; Schreiber et al., 2013; Boschen et al., 2014), though the contribution of immune molecules to these deficits remains to be investigated. Microglia play an important role in synaptic pruning and maintenance, neurogenesis, and apoptosis, and the cytokines expressed by microglia, such as IL-1β, are important mediators of learning-related plasticity (Bilbo & Schwarz, 2009; 2012). During development and adulthood, these processes exist in a balance with optimal levels of cytokines resulting in peak learning performance; deviation from these optimal levels could impair synaptic transmission and maintenance, resulting in reduced long-term potentiation (LTP). Neonatal alcohol exposure has been shown to impair LTP in hippocampal CA1 (Puglia et al., 2010a, b), though a role for cytokines in this deficit has not been established. The long-term

activation status of the microglia and cytokine expression in the alcohol-exposed brain must be investigated. Acknowledging neuroinflammation as a secondary source of damage following neonatal alcohol exposure opens up potential therapeutic possibilities which target the immune response.

Age of the alcohol exposure might play an important role in cytokine response, as previous studies of alcohol exposure in adolescent and adult rats found no change in TNF-α expression (Marshall et al., 2013; McClain et al., 2011; Zahr et al., 2010), while this cytokine was upregulated in the neonatal rodent cerebral cortex, hippocampus, and cerebellum (Drew et al., 2015; Topper et al., 2015). IL-1β gene expression was also increased in all three brain regions in the study from Drew and colleagues (2015), while upregulation was only present in the cerebellum in the report by Topper and colleagues (2015), possibly due to differences in the exposure paradigm and time points used. Drew and colleagues (2015) also reported that CCL2 expression was increased only in hippocampus and cerebellum, showing region-specific profiles of cytokine release following alcohol exposure via intubation. Tiwari and Chopra (2011) found long-lasting increases in both TNF-α and IL-1β levels in the hippocampus and cerebral cortex on PD28 following PD7-9 alcohol exposure. Combined with the results of the current study, it is likely that cytokine expression is enhanced shortly following the alcohol exposure and remains elevated at least until adolescence, suggesting that alcohol-exposed animals may show other signs of central or peripheral immune dysfunction. Various types of stress have been shown to also affect release of cytokines through glucocorticoid signaling pathways, supporting the

increase in cytokine seen in the SI group (Beutler et al., 1986; Minami et al., 1991; Elenkov et al., 1996). The role of stress in the current findings is discussed more in Section 6.4.2.

The current study is one of the first to investigate gene expression levels of an anti-inflammatory cytokine following neonatal alcohol exposure. Expression of anti-inflammatory cytokine TGF-β was increased only in the AE group, suggesting compensatory processes in the brain to minimize long-term alcohol-induced damage through release of anti-inflammatory molecules. Following an insult, TGF-β can reduce production of pro-inflammatory cytokines such as IL-2 and proliferation of T helper immune cells (Su et al., 2012). TGF-β is increased in the liver during alcohol-induced hepatic disease (Meyer et al., 2010; Gerjevic et al., 2012), but its role in the brain's response to alcohol exposure has yet to be fully investigated. Recent work from the Valenzeula group (Topper et al., 2015) found no changes to TGF-β in the hippocampus and cerebellum of rats exposed to alcohol vapor during the neonatal period, possibly indicating an influence of route of administration or timing of analysis on the anti-inflammatory response. The alcohol-specific effect on TGF- β compared to the classic pro-inflammatory cytokines which were upregulated in both AE and SI animals was very intriguing. Glucocorticoids have no effect on levels of the anti-inflammatory cytokine, IL-10, suggesting that stress could play less of a role in TGF-β expression following an insult compared to pro-inflammatory molecule expression (Elenkov et al., 1996).

6.4 General Discussion

6.4.1 Neuroprotection in the Developing Brain

The results of Experiments 1 and 2 support the hypothesis that neonatal alcohol exposure (PD4-9) induces a neuroprotective response in the hippocampus 24 hours following the exposure. In Experiment 1, alcohol exposure uniquely increased levels of the TrkB receptor and exon I-specific *Bdnf* gene expression. These findings, along with the increased BDNF mRNA and protein found in both the AE and SI groups, are consistent with previous literature reporting transiently increased BDNF and NGF levels in the neonatal brain (Heaton et al., 2000; Heaton et al., 2003; Angelucci et al., 1997). Developmental alcohol exposure exacerbates normally-occurring apoptotic processes throughout the brain, with levels of apoptotic cell bodies increasing 350% in hippocampal CA1 on PD5, 24 hours following a single day binge (Gursky et al., *in preparation*). Other models of FASDs have also reported widespread apoptosis in the CNS (Ikonomidou et al., 2000; Dunty et al., 2001; Climent et al., 2002; Olney et al., 2002), likely through silencing neurons via action at NMDA and $GABA_A$ receptors (Lebedeva et al., 2015). BDNF prevents apoptosis in a variety of experimental paradigms, including excitotoxicity and ischemic hypoxia (Almeida et al., 2005; Han et al., 2000). There is also evidence that pharmacological modulation of NGF and BDNF can protect against cytotoxicity and apoptosis following alcohol exposure, though this effect has only been directly demonstrated *in vitro*. Both BDNF and NGF reduced cytotoxicity as measured by a reduction in 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in a model of ethanol exposure

combined with hypoxia and hypoglycemia (Mitchell et al., 1999). Administration of alcohol to cultured human neuroblastomas decreased cell viability (mitochondrial activity) and BDNF and CREB levels; exogenously applied BDNF improved mitochondrial activity in alcohol-exposed cells (Sakai et al., 2005). Alcohol induced apoptosis in cerebellar granule cells, but the upregulation of cell death was blocked by BDNF (Bhave et al., 1999). Finally, Climent and colleagues (2002) showed that BDNF activated anti-apoptotic and pro-cell survival pathways in the postnatal cerebral cortex; however, this activation is disrupted by prenatal alcohol exposure. Further research needs to be done to determine if these results hold true *in vivo*.

The enhancement of TGF-β also represents a potential mechanism of neuroprotection in response to developmental alcohol exposure. Microglia that release anti-inflammatory cytokines, such as TGF-β, are thought to serve a neuroprotective role, and are thus classified as exhibiting an M2, or alternative, microglia phenotype as compared to the classic M1 phenotype associated with the pro-inflammatory response (Olah et al., 2011). Microglia with M2 classification fight inflammation through downregulation of pro-inflammatory factors and mediation of tissue repair (Varin & Gordon 2009). This response is a natural and necessary transition following M1 classical microglial activation and inflammation, continual production of pro-inflammatory cytokines can lead to continued neurotoxicity and cell death if not kept in check (Kigerl et al., 2009). Increased TGF-β gene expression might represent a neuroprotective immune response, as prolonged elevated levels of pro-inflammatory cytokines induce apoptosis and tissue damage. IL-10, a widely studied anti-

inflammatory cytokine, was undetectable in the PD10 hippocampus when we attempted to assess this cytokine in Experiment 2, supporting other literature in human infants showing that the anti-inflammatory response is immature in the neonatal brain and IL-10 might not play a large role in the brain's neuroimmune response during development (Schultz et al., 2004). Recent work by Topper and colleagues (2015) measured levels of IL-10 in the hippocampus and cerebellum of neonatal animals following exposure to alcohol vapor and found no changes in either brain region. However, increased levels of IL-10 have been reported following severe sepsis in human neonates (Ng et al., 2003), indicating that IL-10 response might be restricted to the most severe immune challenges or infections. More work investigating the influence of these anti-inflammatory molecules following developmental alcohol exposure is necessary, in addition to whether exogenous administration offers any protection against alcohol-induced apoptosis and tissue damage.

6.4.2 Role of Intubation Stress in the Current Findings

Stress played an undeniable role in the findings reported in both Experiments 1 and 2. Increased plasma corticosterone was found in both the AE and SI groups compared to undisturbed controls on PD10 (Experiment 1), supporting the notion that intragastric intubation is an inherently stressful procedure. The PD10 age point falls within the "stress hyporesponsive" period, which occurs during the first two weeks of postnatal life and is characterized by low baseline glucocorticoids plasma levels, attenuated hormonal responses to acute stress, impaired negative feedback of circulating corticosterone, and a 3x longer half-life of corticosterone (Lupien et al.,

2009; Sapolsky & Meaney, 1986). In the current study, two main factors could have resulted in the observed increase in corticosterone levels in the AE and SI pups: binge-like alcohol exposure and the stress of the intubation paradigm. While limited literature has examined the short-term impact of postnatal alcohol exposure on plasma corticosterone, neonatal rats prenatally exposed to alcohol are known to exhibit elevated basal levels of corticosterone in plasma and brain and exhibit a blunted stress response (Weinberg, 1989; Taylor et al., 1986). Furthermore, prenatally alcohol-exposed animals are hyper-responsive to stress later in life, which can manifest as hypersecretion of adrenocorticotropic hormone (ACTH) and corticosterone or delayed return to basal levels after acute stressors (Kim et al., 1999). Early-life stress, such as experiencing the intubation procedure, also affects the stress response in neonatal rats, either through short-term increases in corticosterone and ACTH or long-term alterations to the hypothalamic-pituitary-adrenal (HPA) axis (Walker et al., 1991; Weinstock et al., 2009). While short durations of separation or handling (<15 min) speed recovery of corticosterone following a later stressful event (Ader, 1970), prolonged separation increases and extends stress-induced elevations of circulating corticosterone (Levine et al., 1991; Gilles et al., 1996; McCormick et al., 1998). The timing of the increase in corticosterone levels is characteristic of an impaired effective termination mechanism negative feedback system. Thus, early exposures to stressors, such as the intubation procedure, might prime the HPA axis to respond more strongly to minor stressors later in life, such as brief maternal separation or siblings being removed from the home cage (Gilles et al., 1996; McCormick et al., 1998). The close

temporal proximity between the last intubations and sacrifice suggests that stress plays an important role in the interpretation of the results of these studies.

While the data from Experiments 1 and 2 combined suggest that alcohol exposure and sham-intubation can impact the brain similarly on short-term measures that respond to stress as well as alcohol exposure, these findings do not necessarily indicate that stress is the sole driving force behind the overlapping effects. Instead, it is more likely that both alcohol exposure and sham-intubation activate neurotrophins and the neuroimmune system. The neuroinflammatory response, in particular, could present in a similar way independent of the cause (ex. drug exposure, stress, pollution, viral infection). Early life stress has been shown to alter cytokine expression in the neonatal and adult brain. Specifically, two studies using maternal separation stress from PD1-14 showed increased IL-1β concentrations in hippocampus on PD16 (no reported changes to IL-6 and TNF-α) (Roque et al., 2015) and upregulation of both IL-10 and TNF-α in the adult hippocampus (Pinheiro et al., 2015). Interestingly, this manipulation increased microglial activation and decreased number of astrocytes in the neonatal hippocampus (Roque et al., 2015). Both studies show that early life stress paradigms can increase levels of pro- and anti-inflammatory cytokines, dependent on cytokine and timing of tissue analysis. In Experiment 1, both the AE and SI groups show increased levels of plasma corticosterone, indicating that stress likely contributes to the overlapping effects, but the number of effects unique to alcohol exposure (in these experiments, increased TrkB protein levels, *Bdnf* exon I–specific gene expression, microglial cell territory, and expression of CCL4 and TGF-β) discount

stress as the sole factor driving the neurotrophic and neuroinflammatory response in our model.

Beyond its use as a control group for the stress of the alcohol administration procedure, the findings for the sham intubation group has implications for other models of early life stress. In particular, the repeated handling and gavage of third trimester-equivalent pups could be similar to stress related to preterm birth in humans. While many animal models of preterm birth focus on mechanisms that trigger parturition (Elovitz & Mrinalini, 2004), sham intubation could serve as a model of the stress which occurs to the neonate after birth, including maternal separation, insertion of tubes or injections, blood collection, and handling for medical procedures. Preterm infants have increased susceptibility to infection and long-term negative health consequences (Hack & Fanaroff, 1993). Understanding the effect of early postnatal stress on the preterm neonate could give valuable insight as to whether stress during this developmental window has any long-term harmful impact on the infant.

6.5 Conclusion

In summary, third trimester-equivalent binge-like alcohol exposure and intubation stress effect both neuroplasticity and the neuroimmune system in overlapping and distinct ways (Figure 6.11). In Experiment 1, alcohol exposure increases BDNF and TrkB receptor protein in the hippocampus 24 hours following the final alcohol exposure (PD10). Sham intubation results in a similar increase in BDNF protein in the hippocampus, but does not affect TrkB protein expression. *Bdnf* total gene expression is also increased at this time point in both AE and SI pups. The

increase in total gene expression in SI and AE animals might be driven through different promoter regions, as AE animals showed an increase in exon I-specific transcripts while SI animals did not. DNA methylation at this exon was decreased in both AE and SI animals. These data suggest a reactive increase in neurotrophic factor following exposure to a teratogen or intubation stress, potentially as a neuroprotective mechanism. The temporal pattern of the BDNF and TrkB upregulation needs to be further investigated. Basal plasma corticosterone was also elevated in AE and SI animals and the diurnal cycling pattern seems to be disrupted in the AE group. The long-term impact of developmental alcohol exposure on stress hormone function, both at basal levels and in response to novel stressors, remains to be elucidated.

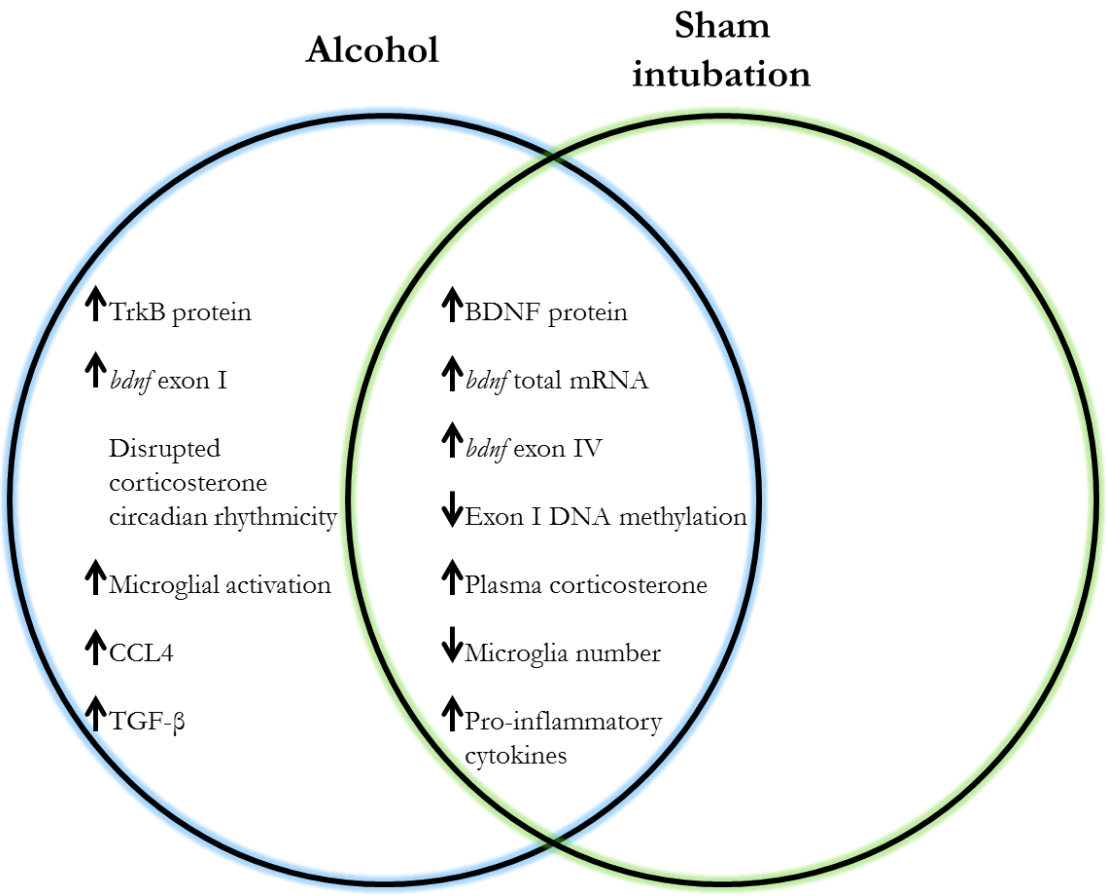

Figure 6.11. Alcohol-specific and Shared Effects of PD4-9 Alcohol Exposure and Sham Intubation in the PD10 Hippocampus.

Experiment 2 found that alcohol exposure on PD4-9 significantly decreases microglial cell number, decreases microglia cell territory indicating enhanced activation, increases pro- and anti-inflammatory cytokine gene expression in the hippocampus on PD10 (Figure 6.11). How long the neuroimmune activation persists following cessation of the alcohol exposure is not known; current work in our lab is pursuing this line of research. Furthermore, how glucocorticoid activation interacts with the neuroimmune response in Experiment 2 remains to be elucidated. The

interaction between the immune system and cognitive function is still being explored, but current evidence supports that alcohol's effects on microglia and cytokine production could have a significant impact on alcohol-related deficits in learning and memory, particularly if there is long-term dysregulation of the immune response or if the individual is faced with another immune challenge later in life. Investigation of long-term neuroimmune function following neonatal alcohol exposure and the effect on cognitive and behavioral outcomes is an essential next step. Importantly, further insults, such as exposure to alcohol or infection during adulthood, would give important information regarding whether early aberrant microglial activation primes later abnormal immune function. In summary, this Experiment adds important information regarding developing immune system and how microglia in the neonatal brain respond to teratogens, such as alcohol exposure.

Continual efforts to understand and refine animal models of human disorders is a critical scientific process. While the intragastric intubation method of alcohol administration is a valid model of FASD on many measures, it's more subtle effects on cells were not originally known and it seems that some neuroplastic and neuroimmune measures may be impacted by stress alone using this technique. The robust effect of stress in these studies were unexpected, but highlight that the potential effects of intubation stress should not be ignored – in fact, the opposite: future studies should investigate how stress might play a role in the observed neuroplastic, neuroimmune or later behavioral changes so the intubation procedure can be altered to reduce stress effects. Overall, these data from this Aim give important insight into how

the brain responds to teratogenic insult in the short-term, as well as critical information regarding how this model of alcohol exposure affects the HPA axis and stress hormone production.

Chapter 7

SPECIFIC AIM 2: INFLUENCE OF AEROBIC EXERCISE ALONE OR FOLLOWED BY ENVIRONMENTAL COMPLEXITY ON HIPPOCAMPAL BDNF EXON-SPECIFIC GENE EXPRESSION, PROTEIN, AND DNA METHYLATION IN ADULT RATS NEONATALLY EXPOSED TO ALCOHOL

7.1 Introduction

Alcohol exposure during the third trimester-equivalent (first two postnatal weeks in rodent models [Dobbing & Sands, 1979]) has a significant impact on the structure and function of late-developing structures such as the hippocampus. In rodent models of FASD that administer alcohol exposure during the third trimester-equivalent, various measures of neuroanatomy and neuroplasticity are impacted: long-lasting decreases in hippocampal CA1 pyramidal cell number (Bonthius & West, 1990), alterations to hippocampal dendritic morphology (Sakata-Haga et al., 2003), decreased survival of newly generated dentate gyrus granule cells (Klintsova et al, 2007), impaired induction of CA1 long-term potentiation (LTP) (Puglia & Valenzuela, 2010a,b), and decreased neuronal activation as measured by expression of the protein c-Fos (Murawski et al., 2012). Deficits in structural and functional plasticity likely contribute to observed impairments on various hippocampal-associated behavioral tasks following neonatal alcohol exposure.

An ongoing challenge for those who research the effect of neonatal alcohol exposure on neuroplasticity is identifying the factors that contribute to impairments in plasticity, as alcohol exposure affects various brain regions, neurotransmitter systems,

and signaling pathways. The experiments in this Aim examine brain-derived neurotrophic factor (BDNF) as a molecular targets impacted by neonatal alcohol exposure and contributing to impaired neuroplasticity in alcohol-exposed rats. Levels of BDNF protein have been shown to be affected by developmental alcohol exposure, though these changes seem to be dependent on alcohol dose, exposure window, brain region and timing of analysis. As discussed in Aim 1 of this dissertation (Chapter 6), PD4-9 alcohol exposure increased BDNF and TrkB protein, *Bdnf* total gene expression and generation of *Bdnf* exon-specific mRNA transcripts, which supported previous work by the Heaton group (2000, 2003) showing increases in hippocampal BDNF protein levels on PD10 immediately following neonatal alcohol exposure. Work investigating BDNF protein in the adult rat brain found that hippocampal BDNF protein levels were decreased in adult rats following moderate (3 g/kg/day) PD7-8 alcohol exposure (Fattori et al., 2008). Miki and colleagues (2008) reported decreased hippocampal *Bdnf* gene expression in the hippocampus on PD60 following alcohol exposure from PD10-15. Overall, these data suggest that developmental alcohol exposure can alter BDNF protein and gene transcription in an age-dependent fashion.

These experiments also investigate two behavioral interventions which are beneficial for neuroplasticity and influence BDNF synthesis: aerobic exercise and housing in a complex environment (EC). Voluntary wheel running (WR) and exposure to EC have been investigated by our research group as promising therapies to mitigate the structural, functional, and behavioral impairments caused by neonatal alcohol exposure. Both WR and EC have been shown to have robust benefits on measures of

neuroplasticity and learning in the normal rodent brain, including increased hippocampal dendritic complexity, angiogenesis, upregulation of c-Fos, enhanced LTP and improved performance on hippocampal-associated spatial tasks. In addition, our lab has demonstrated that 12 days of WR followed by 30 days of EC increases the survival of newly generated dentate gyrus granule cells in animals neonatally exposed to alcohol. Comparatively, alcohol-exposed animals housed in 12 days of WR followed by 30 days of standard housing (SH) showed impaired new cell survival (Hamilton et al., 2012; Helfer et al., 2009). Disruptions to adult neurogenesis could contribute to behavioral impairments observed in rodent models of FASD (Bruel-Jungerman et al., 2007; Sahay et al., 2011). Research has shown that WR and EC affect neuroplasticity through distinct yet overlapping mechanisms (Olson et al., 2006) and that EC paradigms that include a component of aerobic exercise are the most beneficial (Mustroph et al., 2012; Kobilo et al., 2013). In particular, both WR and EC increase protein and mRNA levels of BDNF in both humans and rodents (Berchtold et al., 2005; Christie et al., 2005; Griffin et al., 2009; Kuzumaki et al., 2011; Rasmussen et al., 2009). Importantly, as described previously, BDNF is critical for measures of neuroplasticity that are shown to be negatively impacted by neonatal alcohol exposure, suggesting that long-term alterations to BDNF expression might underlie both alcohol-induced deficits in neuroplasticity and the beneficial effects of WR/EC in neonatally alcohol-exposed adult animals.

The goal of Specific Aim 2 was to extend the results regarding BDNF from Aim 1 (neonatal time point) by assessing *Bdnf* total and exon-specific gene expression

and BDNF protein in the hippocampus of adult rats (PD72) neonatally exposed to alcohol (PD4-9, 5.25 g/kg/day). In addition, Aim 2 examines *Bdnf* DNA methylation at PD72, as DNA methylation status can be dynamic across the lifespan and it is possible that the decreases observed on PD10 were transient. It is equally possible that the observed decreases could be long-lived (i.e. present in both age groups) or a different pattern could be present by PD72. Finally, Aim 2 tested the hypothesis that exposure to either a "super-intervention" [voluntary wheel running (12 days) followed by housing in a complex environment (30 days)] or continuous wheel running (42 days) alters *Bdnf* gene expression, BDNF protein levels, and DNA methylation. The use of two different behavioral interventions (continuous aerobic vs. aerobic followed by social and behavioral enrichment) allowed for the assessment of whether exercise alone is sufficient to enhance hippocampal BDNF.

7.2 Materials and Methods

For detailed information regarding the subjects, materials and methods used, see Chapter 5 (General Procedures). Methods will be briefly described below with details pertaining to the specific experiments in this Aim.

7.2.1 Subjects

Briefly, timed pregnant Long-Evans dams were obtained (Harlan Laboratories, Indianapolis, IN). On postnatal day (PD) 3, litters were culled to eight pups each (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). Following the alcohol exposure procedure, pups were left undisturbed with the

dam until weaning (PD23). On PD23, rats were placed in standard cages in groups of 3 same-sex animals, counterbalanced for litter and neonatal condition. They remained in these cages until PD30, when each cage was assigned to one of three housing conditions, described below in 7.1.1.3. In total, 83 male rat pups were generated for the current study (15 litters). The specific sample sizes for each experimental condition used for each assay are listed below.

7.2.2 Alcohol Exposure and BAC analysis

On PD4-9, AE pups were exposed to alcohol in a binge-like manner (5.25 g/kg/day) via intragastric intubation (Figure 7.1). A total of 3 male pups died during or shortly following the intubation procedure. On PD4, blood samples were obtained from AE pups via tail clip for BAC analysis 90 minutes following the second alcohol exposure. Plasma was analyzed for BAC using an Analox GL5 Alcohol Analyzer (Analox Instruments, Boston, MA). Due to a machine malfunction, plasma from a subset of animals from these experiments was not able to be analyzed (8 animals).

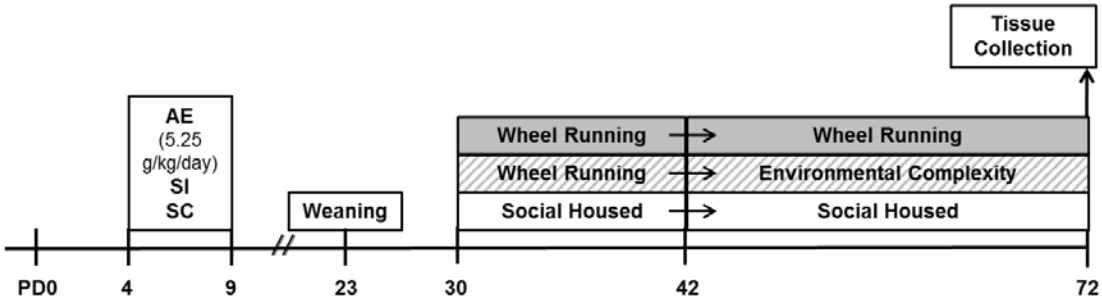

Figure 7.1. Experimental Timeline.

PD: Postnatal Day; AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control.

7.2.3 Adolescent and Adult Housing Conditions

On PD30, each cage was assigned one of three adolescent/adult housing conditions: standard social housing (SH), continuous access to wheel running for 42 days (WR/WR), or 12 days of wheel running access followed by 30 days of housing in a complex environment (WR/EC) (Figure 7.1). Animals were weighed approximately every 8 days and sacrificed on PD72.

Standard social housing (SH) consisted of three rats housed in opaque cages. Wheel running (WR) consisted of rats housed in cages with 24 h voluntary access to running wheels attached to standard sized cages. For SH and WR, animals were housed 3 per cage in the same configuration as PD23-30 housing. Running distance was recorded daily at 9 AM. Rats were housing in this condition either for 42 days (PD30-72) or for 12 days (PD30-42) prior to housing in EC (PD42-72). Each EC cage consisted of a 3-story cage with three ramps, two balconies and a full middle floor. Each cage housed 9-12 male animals (3-4 animals/neonatal condition) and was equipped with a variety of toys, large tubes, and buckets. The toys were changed every 2nd day. Animals were taken directly from their housing condition for sacrifice on PD72.

7.2.4 Gene Expression Analysis

Animals were briefly anesthetized with isoflurane and rapidly decapitated with a guillotine. The brain was flash frozen with 2-Methylbutane and sectioned on a brain matrix (1 µm). Sections were stored at -80°C. The hippocampus (both dorsal and ventral) was dissected on dry ice and homogenized. DNA/RNA were extracted and

reverse transcription was performed to generate cDNA from the RNA. cDNA was amplified by real-time PCR (Bio-Rad CFX96) with Taqman probes (Applied Biosystems) to target *Bdnf* total mRNA (exon IX) and exon I- and IV-specific transcripts. *Tubulin* was used as a reference gene. All reactions for each gene target and reference were run in triplicate. Product specificity was verified using gel electrophoresis. The final animal number used for this assay was 7-11/group.

7.2.5 BDNF Protein Analysis

Animals were sacrificed via rapid decapitation on PD72 directly from their housing condition. The Chemikine BDNF Sandwich ELISA kit (Millipore, Billerica, MA) was used. Total brain protein was detected with Coumassie Protein Assay (ThermoScientific). Data were expressed as pg target protein per mg of total brain protein for tissue and pg corticosterone per mL for plasma. Results were then combined as percent of control between plates. Final sample sizes for this assay were 7-8/group.

7.2.6 DNA Methylation Analysis

Methylation status of *Bdnf* exon I was assessed using methylation specific real-time PCR (MSP) on bisulfite-converted DNA (Qiagen Inc., Valencia, CA). This exon was chosen to remain consistent with the neonatal time point (Specific Aim 1), where alcohol-specific increases in exon I-driven gene expression were reported. Primer sets (Sigma-Aldrich; listed below in Table 1; Roth et al., 2009; Roth et al., 2015) targeted methylated and unmethylated CG dinucleotides in DNA associated with *Bdnf* exon I or *tubulin* (used as a reference gene). All reactions were run in triplicate. Primer

specificity was determined by melt curve analysis on all samples and gel electrophoresis on a sub-set of samples. The final sample size for these analyses was 5-7/group.

Primer	Sequence (5' to 3')
***Bdnf* exon I Methylated**	Forward: CGGAAAGTATTAGAGGTAGGGTAGC Reverse: TACGAACCCTAAATCTCTAAACGAA
***Bdnf* exon I Unmethylated**	Forward: TGGAAAGTATTAGAGGTAGGGTAGTGA Reverse: TACAAACCCTAAATCTCTAAACAAA
Tubulin	Forward: GGAGAGTAATATGAATGATTTGGTG Reverse: CATCTCAACTTTCCCTAACCTACTTAA

Table 7.1. Custom Primer Sets for MSP Analysis.

7.2.6 Statistical Analyses

Weights for each day of dosing (PD4-9) and housing (PD30, 42, 50, 58, 66, and 72) were averaged across neonatal condition/housing condition for each day. PD4-9 weights were analyzed using a repeated-measures analysis of variance (ANOVA) and PD30-72 weights were analyzed with a two-way repeated-measures ANOVA followed by *post hoc* tests when appropriate. For blood alcohol concentrations, average PD4 BACs for each animal (two or three analyses run per animal) ± standard error of the mean (SEM) are reported as mg/dl. For BDNF protein data, pg target protein/mg total brain protein data were transformed into percent of control between plates to control for differences in the ELISA kit standards. Inconsistent optical density values (>30% difference between duplicate wells) were removed from the analyses. Differences in BDNF protein data were analyzed using a two-way ANOVA with appropriate *post hoc* tests run as needed (Tukey's). For gene expression and MSP

data, the comparative Ct method was used to obtain the relative fold change of experimental (AE or SI) vs. the average of controls (SC) per plate (Livak and Schmittgen, 2001). Two-way ANOVAs were run on gene expression and MSP data, followed by Tukey's *post hoc* tests when appropriate. Differences were considered to be statistically significant at $p < 0.05$ and nonsignificant trends at $p < 0.1$ are also reported. For all studies, outliers were identified using Grubb's test. Two-way ANOVAs were run using SPSS (v14) or Prism 6 software (GraphPad Inc.).

7.3 Results

7.3.1 Body Weights, BACs, and Running History

PD4-9 weights were analyzed with a repeated-measures ANOVA. An interaction between Day and Neonatal Condition was found ($F_{(10,480)} = 2.375$, $p = 0.0095$), as well as main effects of Day ($F_{(5, 480)} = 404.5$, $p < 0.0001$) and Neonatal Condition ($F_{(2,480)} = 33.42$, $p < 0.0001$) (Figure 7.2). Tukey's multiple comparison *post hoc* determined that on PD5, AE animals weighed less than SI pups ($p < 0.05$), and that for days 6-9, AE pups weighed less than both SI and SC pups ($p < 0.05$ in all cases).

For PD30-72, a Day x Housing interaction was found ($F_{(10,370)} = 2.682$, $p = 0.004$), as well as a main effect of Day ($F_{(5,370)} = 2022.357$, $p < 0.0001$) and a trending Day x Neonatal interaction ($F_{(10,370)} = 1.696$, $p = 0.08$). The interaction between Day, Neonatal Condition, and Housing was not significant ($p > 0.1$). *Post hoc* tests (Tukey's HSD) revealed a trending decrease in weight for WRWR animals compared to the SH group ($p = 0.099$); WREC and SH animals did not differ. There was a

significant decrease in weight for AE animals compared to SC ($p = 0.042$), while SC and SI did not differ. Long-lasting changes to body weight due to alcohol exposure have never been found previously using our model or in Aim 3 of this dissertation, leading us to conclude that this finding is due to unknown factors specific to this cohort.

BACs were analyzed from plasma collected using a tail clip on PD4, 2 hours following the second alcohol exposure. The average BAC was 321.6 ± 23.6 mg/dl, which is in accordance with previously published BACs using our model (Hamilton et al., 2012; Boschen et al., 2014).

Running distance per 24 hours was measured per cage (3 animals/cage). For the first 12 days of wheel running access (PD31-42), average running distance was 2.57 miles (± 1.13 SD) per 24 hours. For WRWR animals, the average distance ran from PD43-72 was 7.75 miles (± 3.94 SD) per 24 hours. These running distances are consistent with previously published work from our lab using the WREC intervention (Hamilton et al., 2012; Boschen et al., 2014). The increase in running during the last 30 days in WRWR animals compared to the first two days could be due to the longer length/stride of the animals as they aged or natural increases in running endurance.

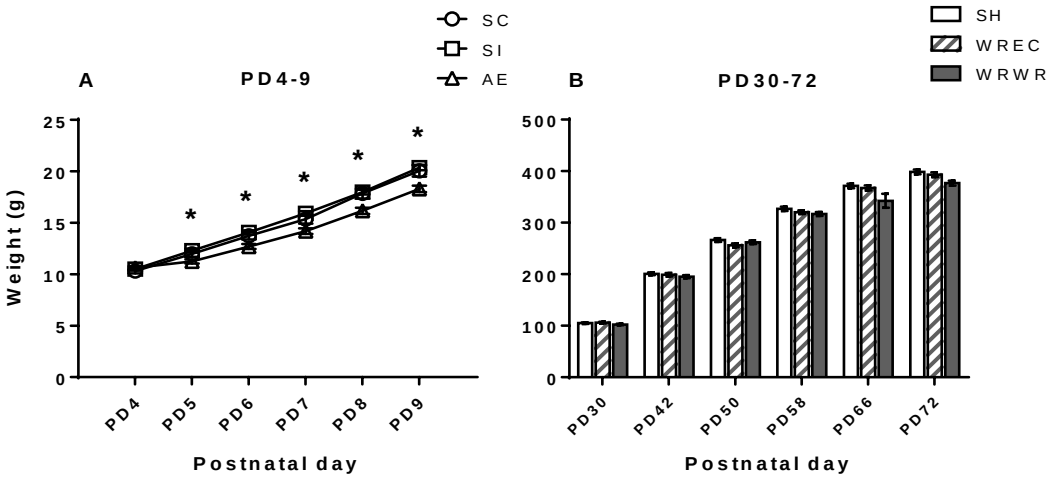

Figure 7.2. Postnatal Body Weights Across Conditions.

A) PD4-9 weights across the three neonatal conditions. For PD5, AE differed from SI, but differed from SC and SI on PD6-9. B) PD30-72 weights by housing condition (collapsed across neonatal condition). * = $p < 0.05$. AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control; SH: Standard social housing; WREC: Wheel running followed by environmental complexity; WRWR: Wheel running.

7.3.2 Gene Expression

Total *Bdnf* gene expression (by targeting all exon-IX containing transcripts) was measured in the hippocampus on PD72. A main effect of housing was found using a two-way ANOVA ($F_{(2,71)} = 24.57$, $p < 0.0001$), but no effect of neonatal condition or housing was revealed. Total *Bdnf* gene expression was increased in the WRWR condition in all neonatal conditions ($p < 0.05$; Figure 7.3A). For all neonatal treatments, gene expression was increased in WRWR compared to both SH and WREC ($p < 0.05$). In all conditions, WREC and SH did not differ from one another. We also examined expression of *Bdnf* exon I- and IV-specific transcripts. For exon I-specific transcripts, a main effect of housing was found ($F_{(2,73)} = 33.08$, $p < 0.0001$),

but no effect of neonatal condition or housing was observed. Gene expression was increased in the WRWR condition compared to both SH and WREC in all neonatal conditions ($p < 0.05$, Figure 7.3B). WREC and SH did not differ from one another. For exon IV-specific transcripts, again, a main effect of housing was found ($F_{(2,72)} = 19.53$, $p < 0.0001$), but no effect of neonatal condition or housing was found. Gene expression was increased by WRWR compared to both SH and WREC in both SC and AE ($p < 0.05$, Figure 7.23). For SI, a trend was found that WRWR had higher exon IV gene expression compared to SI was found ($p < 0.1$). In all conditions, WREC and SH did not differ from one another.

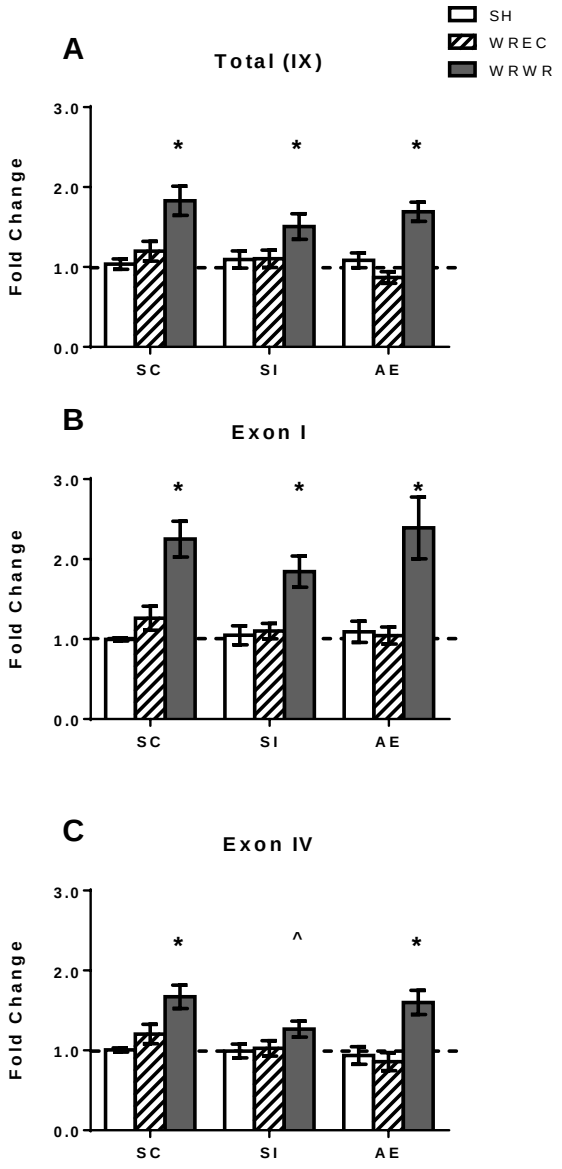

Figure 7.3. *Bdnf* gene expression in the PD72 hippocampus.

A) WRWR showed more total *Bdnf* gene expression compared to SH and WREC animals. B) and C) Exon I- and IV-specific transcripts were elevated in all WRWR animals. For SI animals, a trending increase was found for exon IV-specific gene expression in WRWR pups relative to SH ($p < 0.1$, indicated as ^ on graph). * $p < 0.05$. Data is expressed as a fold change from the SH-suckle control (SC) group (shown as 1 on graphs). AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control; SH: Standard social housing; WREC: Wheel running followed by environmental complexity; WRWR: Wheel running.

7.3.3 BDNF Protein Levels

BDNF protein levels were measured from PD72 hippocampal tissue. A two-way ANOVA revealed a main effect of housing ($F_{(2,57)} = 9.268$, $p = 0.0003$, Figure 7.4). *Post hoc* tests (Tukey's multiple comparison test) showed that for SC, BDNF protein expression was significantly increased in the WRWR group compared to SH and WREC ($p < 0.05$). In SI animals, no significant effects were found. For AE, WRWR significantly increased BDNF protein levels compared to the WREC group only ($p < 0.05$). In all conditions, WREC rats did not differ from SH animals.

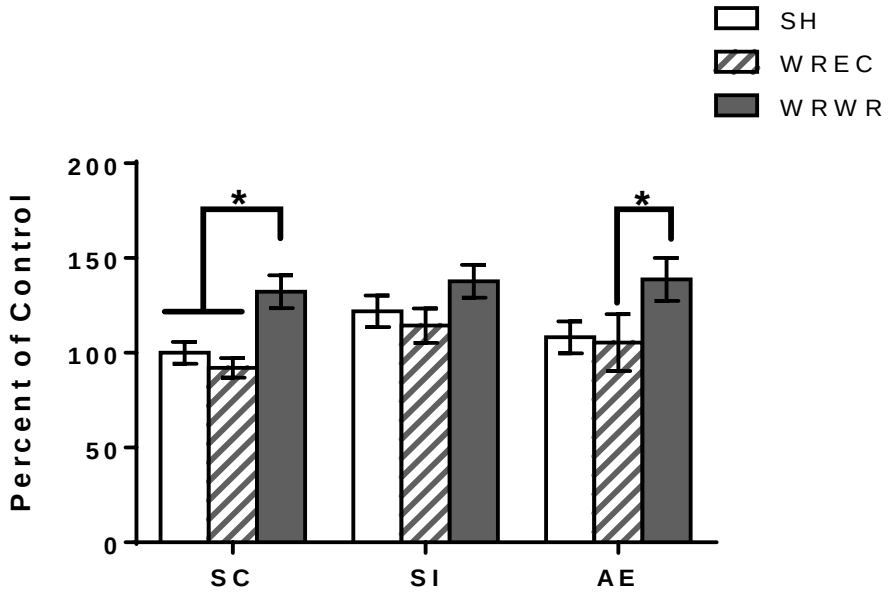

Figure 7.4. BDNF protein in the hippocampus on PD72.

For SC animals, WRWR increased BDNF compared to SH and WREC animals. AE rats had more BDNF following WRWR compared to WREC. * $p < 0.05$. Data is shown as mean percent of control ± SEM. AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control; SH: Standard social housing; WREC: Wheel running followed by environmental complexity; WRWR: Wheel running.

7.3.4 *Bdnf* Exon I DNA Methylation

Bdnf exon I DNA methylation was analyzed in the PD72 hippocampus. A two-way ANOVA found a significant main effect of housing ($F_{(2,53)} = 4.838$, $p = 0.0118$, Figure 7.5). No main effect of neonatal condition or interaction was found. Tukey's *post hoc* analysis revealed that in the SC and SI groups, WRWR was associated with less DNA methylation at exon I ($p < 0.05$) compared to SH. Levels of methylation in the WREC and SH were similar. No significant changes to levels of methylation were found for the AE group.

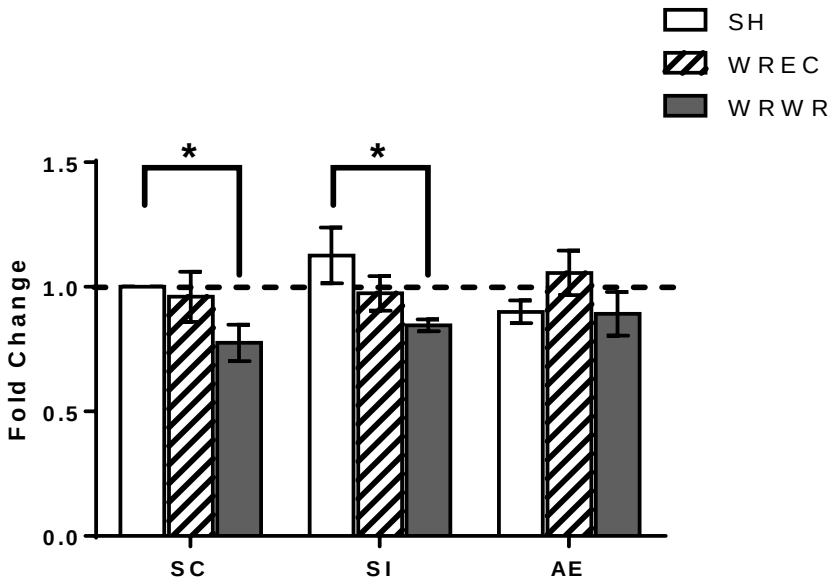

Figure 7.5. *Bdnf* exon I DNA methylation in the PD72 hippocampus.

WRWR rats showed significantly less *Bdnf* DNA methylation at exon I compared to SH in the SC and SI groups. * $p < 0.05$, *** $p < 0.001$. Data is expressed as a fold change ± SEM from the SC/SH group (shown as 1 on graphs). AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control; SH: Standard social housing; WREC: Wheel running followed by environmental complexity; WRWR: Wheel running.

7.4 Discussion

This experiment demonstrated that 1) the influence of third trimester-equivalent (PD4-9) binge-like alcohol exposure (AE) on the basal expression of BDNF is transient, as no effect of neonatal condition was found on PD72, and 2) continuous wheel running (WRWR) and wheel running followed by environmental complexity (WREC) have differential effects on BDNF expression in the adult rat hippocampus. To our knowledge, these results are the first to examine BDNF mRNA, protein levels, and *Bdnf* DNA methylation in adulthood within one model of alcohol exposure or comparing wheel running and housing in EC on these measures. At PD72, AE did not alter hippocampal *Bdnf* gene expression, protein levels or DNA methylation in the social housed (SH) condition. In all groups, WRWR but not WREC upregulated BDNF, though this effect varied slightly across measures and neonatal treatments. Exon I DNA methylation was also decreased by WRWR in the control animals, showing that aerobic exercise can cause epigenetic modifications at this specific *Bdnf* gene locus. Overall, when combined with alterations to BDNF reported in Specific Aim 1, these findings suggest that exposure to alcohol during development has a transient impact on hippocampal BDNF in baseline conditions (SH) and that our two behavioral interventions affect neuroplasticity through different pathways, one mediated by BDNF and one independent of BDNF enhancement.

The findings in the SC group were consistent with the expected relationship between DNA methylation, gene expression, and protein levels. SC animals showed decreased *Bdnf* exon I methylation which corresponded with increased exon I-driven

gene expression. Total *Bdnf* gene expression was also increased in the group and levels of BDNF protein were significantly upregulated as well. While this relationship was not always clear cut in the SI and AE groups, it is beyond the scope of this dissertation to directly examine the molecular mechanisms contributing to the differences in methylation, gene expression, and protein levels in the neonatal treatments. A variety of factors could influence our findings at each step. Other epigenetic modifications, including DNA hydroxymethylation or histone acetylation, could account for inconsistencies between methylation patterns and gene expression. Furthermore, methylation be unassociated with changes to baseline gene expression but instead indicate that the transcriptional system is primed to respond to stimulation (Li et al., 2014). DNA methylation could affect gene transcription beyond altering the ability of transcription factors to bind to promoter regions, including control of alternative splicing, insertion or deletion of transposable elements, or shortening the distance between nucleosomes (Baker-Andresen et al., 2013). In addition, methylation of other exons could be altered, which is a target for future research. Posttranscriptional regulation of RNA could be influenced by the presence of small interfering or microRNAs (siRNAs or miRNAs) causing degradation of the RNA or inefficient translation into protein. Further work is needed to determine if any of these factors are applicable to the findings reported here.

The differential effect of WRWR and WREC on BDNF in the current study is very interesting, as both of these interventions are beneficial for synaptic plasticity and are reported to upregulate BDNF in the literature (Cotman & Berchtold, 2002; Marlatt

et al., 2012; Vaynman et al., 2004; Rasmussen et al., 2009; Torasdotter et al., 1996, 1998; Pham et al., 1999; Ickes et al., 2000). In our laboratory, WREC rescued adult-born granule cell survival in the dentate gyrus, improved contextual fear and trace eyeblink conditioning in AE rats, and increased dendritic complexity in the prefrontal cortex (Hamilton et al., 2011, 2012, 2014, 2015; Schreiber et al., 2013). Thus, this intervention is beneficial for neuroplasticity and learning independent of changes to BDNF at PD72. It is possible that BDNF was upregulated by the 12 days of wheel running from PD30-42 and then, following placement in the EC cage, decreased to baseline while maintaining changes to synaptic plasticity which were initiated during wheel running. Additional experimental time points (specifically, PD42) would be necessary to address this question.

Both exercise and EC have been reported as altering *Bdnf* DNA methylation (Gomez-Pinilla et al., 2011; Kuzumaki et al., 2011). Gomez-Pinilla and colleagues (2011) found that 7 days of wheel running reduced *Bdnf* promoter IV methylation in the rat hippocampus, consistent with the results reported here for exon I. Kuzumaki's group (2011) reported that housing in an complex environment for 4 weeks increased H3K4 histone trimethylation (a marker for active transcription) at promoters III and VI and decreased H3K9 histone trimethylation (a marker for transcriptional repression) at the promoter regions for exons IV and VI in the mouse hippocampus. However, their EC paradigm included running wheels so without a control groups either housed in EC without access to wheels or in standard cages with a wheel, it is possible that aerobic exercise is driving the effect on histone methylation.

Previous work from our lab (Specific Aim 1) found that PD4-9 alcohol exposure decreased *Bdnf* exon I DNA methylation, an effect which was also present in SI animals. Interestingly, neither group showed altered methylation at PD72, suggesting that this epigenetic modification was transient. Previous work showing that developmental alcohol exposure increased DNA methylation and DNA methyltransferase activity in the hippocampus have focused on earlier time points (PD21) (Otero et al., 2012; Perkins et al., 2013). While these studies did not assess *Bdnf* in particular, age of the animal could significantly influence gene-specific methylation. Models of early life stress have shown similar patterns of age-dependent epigenetic modifications (Roth et al., 2009; Blaze et al., 2013). Exposure to an aversive maternal care environment from PD1-7 altered *Bdnf* I and IV methylation patterns dependent on age and sex in the medial prefrontal cortex (Blaze et al., 2013). Specifically, male animals in the maltreated condition showed no methylation changes at PD8, increased methylation at exon I on PD30, and decreased exon I methylation on PD90. No changes to Exon IV methylation were found. This data supports the use of a lifespan approach when assessing DNA methylation patterns.

Interpretation of the current data must also be kept in the context of the tissue and cell-type examined, as methylation patterns can vary drastically between neuronal and non-neuronal cells (Iwamoto et al., 2011). These experiments examined whole hippocampus, including dorsal and ventral regions and all cell types. Thus, the results might have been different if dentate gyrus, CA3, and CA1 were analyzed separately. In addition, the cells of the hippocampus are heterogeneous and includes granule and

pyramidal neurons, a variety of inhibitory interneurons, and glial cells. Both neurons and astrocytes synthesize BDNF (Miklic et al., 2004); the use of fluorescence-activated cell sorting (FACS) to separate neuronal and non-neuronal cells prior to analysis would give valuable information about BDNF levels in different cell types. Levels of the high-affinity BDNF receptor TrkB were not able to be assessed in this experiment due to ELISA kit failure, but this analysis remains a goal of future work in the lab. While levels of BDNF protein were not altered in AE animals, these results do not exclude alterations to posttranslational regulation of BDNF, including cleavage from proBDNF to the mature isoform or trafficking of the protein for activity-dependent release. Levels of the p75 receptor, which binds neurotrophins and activates pro-apoptotic pathways, should also be evaluated. Finally, other plasticity-related molecules likely are involved with the alcohol-related deficits in hippocampal anatomy and function. Prime candidates include other growth factors (e.g. NGF, VEGF, IGF) or immediate early genes such as early growth response protein (EGR-1 of Zif268) or activity-regulated cytoskeleton-associated protein (Arc). Investigation of these proteins is necessary, as well as analysis of other brain regions involved in the behavioral dysfunction seen in rodent models of FASD such as the prefrontal cortex and amygdala.

7.5 Conclusions

In summary, third trimester-equivalent binge-like alcohol exposure does not alter baseline BDNF protein, total or exon-specific gene expression, or exon I DNA methylation in the hippocampus on PD72, suggesting that the effect of PD4-9 alcohol

exposure on BDNF is transient. WRWR increased BDNF protein and gene expression and decreased exon I DNA methylation, while WREC had no effect on these measures. These data give important information about how our behavioral interventions impact synthesis of a critical growth factor involved in a variety of plasticity-related processes. While these experiments point to many more avenues of research which must be explored, they are also the first to assess multiple levels of BDNF synthesis using the same model of alcohol exposure and a lifespan approach. Ultimately, these data support continuing to investigate mechanisms underlying the beneficial effects of exercise and EC on alcohol-induced deficits.

Chapter 8

SPECIFIC AIM 3: INFLUENCE OF AEROBIC EXERCISE ALONE OR FOLLOWED BY ENVIRONMENTAL COMPLEXITY ON HIPPOCAMPAL ADULT NEUROGENESIS IN RATS NEONATALLY EXPOSED TO ALCOHOL

8.1 Introduction

Postnatal models of FASD significantly impact the long-term structure and function of late-developing structures such as the hippocampus. One measure of hippocampal neuroplasticity which has been consistently shown to be affected by developmental alcohol exposure is the process of adult neurogenesis (Ieraci and Herrera, 2007; Singh et al., 2009; Gil-Mohapel et al., 2011). Specifically, our lab has found that PD4-9 alcohol exposure disrupts the survival of adult-born dentate gyrus granule cells 30 days following incorporation of bromodeoxyuridine (BrdU) into the proliferating cell population, while not affecting the generation of new cells (Klintsova et al., 2007). Disruptions to adult neurogenesis could contribute to behavioral impairments observed in rodent models of FASD (Thomas et al., 2008; Hamilton et al., 2011, 2014; Murawski et al., 2012).

Prior to successful integration of newly generated neurons into the hippocampal circuit, these cells undergo a process known as “competitive survival” (Kempermann, 2011). Competitive granule cell survival occurs following the initiation of glutamatergic input to these neurons from the medial entorhinal cortex via the perforant pathway. During this maturation stage, granule cells have a low threshold for excitability due to their small size and higher resting membrane potential (van Praag et

al., 2002; Overstreet-Wadiche & Westbrook, 2006; Kempermann, 2011). Neurons must make a sufficient number of excitatory synaptic connections during this period to successfully integrate and survive. Based on our lab's previous work, we hypothesized that new dentate gyrus neurons in alcohol-exposed animals have a lower rate of cells which successfully make it through competitive survival, leading to the decrease in surviving neurons we observe with our model of FASD. One way to determine the health of new neurons going into the period of competitive survival is to assess dendritic morphology of immature neurons in the dentate gyrus. If these immature neurons exhibit decreased dendritic complexity, the ability to make sufficient number of synaptic connections may be impaired.

Voluntary wheel running (WR) and exposure to a complex environment (EC) have been shown to have robust benefits on hippocampal adult neurogenesis and dendritic morphology in the hippocampus and prefrontal cortex (Kronenberg et al., 2006; Van de Borght et al., 2007; Faherty et al., 2003; Gelfo et al., 2009). Our lab has demonstrated that 12 days of WR followed by 30 days of EC increases the survival of newly generated dentate gyrus granule cells in animals neonatally exposed to alcohol (Hamilton et al., 2012, 2014). Comparatively, alcohol-exposed animals housed in 12 days of WR followed by 30 days of standard housing (SH) showed impaired new cell survival (Helfer et al., 2009).

This Aim sought to: 1) replicate previous work showing that binge-like alcohol exposure on PD4-9 does not affect the number of proliferating cells in the adult rat dorsal dentate gyrus and 2) assess whether PD4-9 alcohol exposure impacts the

number and dendritic morphology of immature neurons in the dentate gyrus. This Aim also investigates the impact of two behavioral interventions, exercise alone or exercise followed by housing in a complex environment, on these measures of hippocampal adult neurogenesis. As spatial and contextual memory deficits are consistently observed following neonatal alcohol exposure (Thomas et al., 2008; Murawski et al., 2012; Schreiber et al., 2013), this Aim focuses on adult neurogenesis in the dorsal dentate gyrus due to its more prominent role in spatial navigation and contextual memory compared to the ventral hippocampus which is more involved with emotional processing due to strong connections with the amygdala (Fanselow & Dong, 2007).

8.2 Materials and Methods

For detailed information regarding the subjects, materials and methods used, see Chapter 5 (General Procedures). Methods will be briefly described below with details pertaining to the specific experiments in this Aim.

8.2.1 Subjects

Briefly, timed pregnant Long-Evans dams (Harlan Laboratories, Indianapolis, IN) were obtained. Litters were culled to eight pups each (6 male, 2 female when possible) on postnatal day (PD) 3. On PD4, pups were assigned to one of three experimental groups: suckle control (SC), sham-intubated (SI) or alcohol exposed (AE). Following the alcohol exposure paradigm, pups and dam were left undisturbed until weaning (PD23). On PD23, rats were housed in standard cages in groups of 3 same-sex individuals, counterbalanced for litter and neonatal condition, where they remained until PD30. On PD30, each cage was assigned to one of three housing

conditions, described below in 8.2.3. In total, 64 male rat pups were generated for this experiment (11 litters). The specific sample sizes for each experimental condition used for each measure are listed below.

8.2.2 Alcohol Exposure and BAC Analysis

On PD4-9, AE pups were exposed to alcohol in a binge-like manner (5.25 g/kg/day) via intragastric intubation (Figure 8.1). One male pup died during the intubation procedure. On PD4, blood samples were obtained from AE pups via tail clip for BAC analysis 90 minutes following the second alcohol exposure. Plasma was analyzed for BAC using an Analox GL5 Alcohol Analyzer (Analox Instruments, Boston, MA).

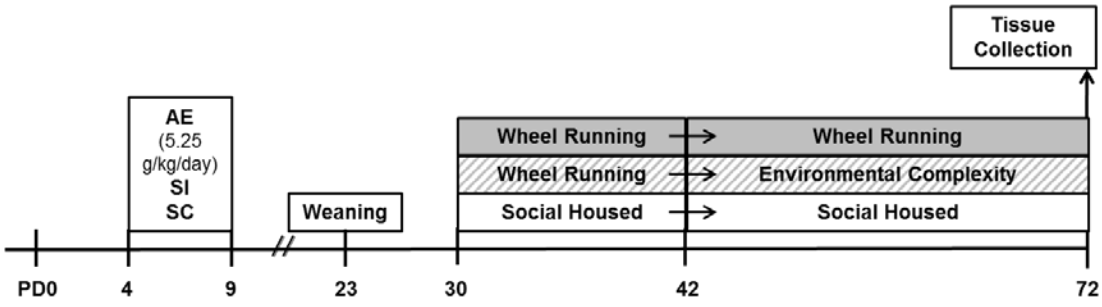

Figure 8.1. Experimental Timeline.

PD: Postnatal Day; AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control.

8.2.3 Adolescent and Adult Housing Conditions

On PD30, each cage was assigned one of three adolescent/adult housing conditions: standard social housing (SH), continuous access to wheel running for 42

days (WRWR), or 12 days of wheel running followed by 30 days of housing in a complex environment (WREC) (Figure 8.1). Animals were sacrificed on PD72.

Standard social housing (SH) consisted of three rats housed in standard opaque cages. Wheel running (WR) consisted of 3 rats housed in cages with 24 h voluntary access to running wheels attached to standard-sized opaque cages. Running distance per 24 h period was recorded daily at 9 AM. Rats were housing in this condition either for 42 days (PD30-72) or for 12 days (PD30-42) prior to housing in EC (PD42-72). Each EC cage consisted of a large, 3-story cage with three ramps, two balconies and a full middle floor. Each EC cage housed 9-12 male animals (3-4 animals/neonatal condition) and a variety of novelty items and shelters. Objects were changed every 2nd day. Animals were taken directly from their housing condition for sacrifice on PD72.

8.2.4 Immunohistochemistry

On day of sacrifice, animals were deeply anesthetized (ketamine/xylazine cocktail), transcardially perfused (0.1M PBS with heparin followed by 4% paraformaldehyde) and the brains stored in 30% sucrose in 4% paraformaldehyde at 4°C. Brains were sectioned horizontally at 40 µm through the entirety of the hippocampus. Immunohistochemistry was performed on a pseudo-randomly selected set of section (1/16th or 1/8th) which included the dorsal dentate gyrus (~-3.1 to -4.3 mm DV bregma). Sections were then mounted onto gelatinized slides, dried for at least 24 hours, and counterstained with Pyronin Y and coverslipped with DPX.

8.2.4.1 Ki-67 Immunohistochemistry

Ki-67 was used to label proliferating progenitor cells in the dorsal hippocampal dentate gyrus (Figure 8.2). Ki-67+ cells are actively going through the cell cycle. Only dorsal dentate gyrus (~-3.1 to -4.3 mm DV bregma) was included with a sampling ratio of every 8th section, meaning that 5-6 sections were immunolabeled per animal (n = 4-6/group). The protocol used was:

1. Wash tissue in 0.1M Tris-buffered saline (TBS) 3 x 5 min
2. Incubation in 0.6% H2O2 in 0.1M TBS for 30 min
3. Wash tissue in 0.1M TBS 3 x 5 min
4. Incubation in blocking solution (3% normal goat serum + 0.1% Triton-X100 + 2% BSA made in 0.1M TBS) for one hour at room temperature
5. Incubation of sections in primary antibody (rabbit anti-Ki67, 1:500 dilution, Abcam cat. #16667) in washing solution (3% normal goat serum + 2% BSA in 0.1M TBS) for 24 hours at 4°C
6. Wash tissue in 0.1M TBS for 3 x 5 min
7. Wash sections in washing solution for 2x 10 min
8. Incubate sections in secondary antibody (goat anti-rabbit biotinylated secondary, dilution 1:500, Vector) in washing solution for 1-2 hours at room temperature
9. Wash tissue in 0.1M TBS 2 x 10 min
10. Wash tissue in washing solution 1 x 10 min

11. Incubate tissue in Avidin-Biotin Complex solution (Vector) mixed in washing solution for one hour at room temperature
12. Wash tissue in 0.1M TBS 3 x 5 min
13. Visualize immunolabeling with 0.5 mg/ml nickel-enhanced diaminobenzedine (DAB) and 0.33 µl/ml H_2O_2 in 0.1M TBS
14. 0.1M TBS rinse for 1-2 sec
15. Wash tissue in 0.1M TBS 2 x 5 min

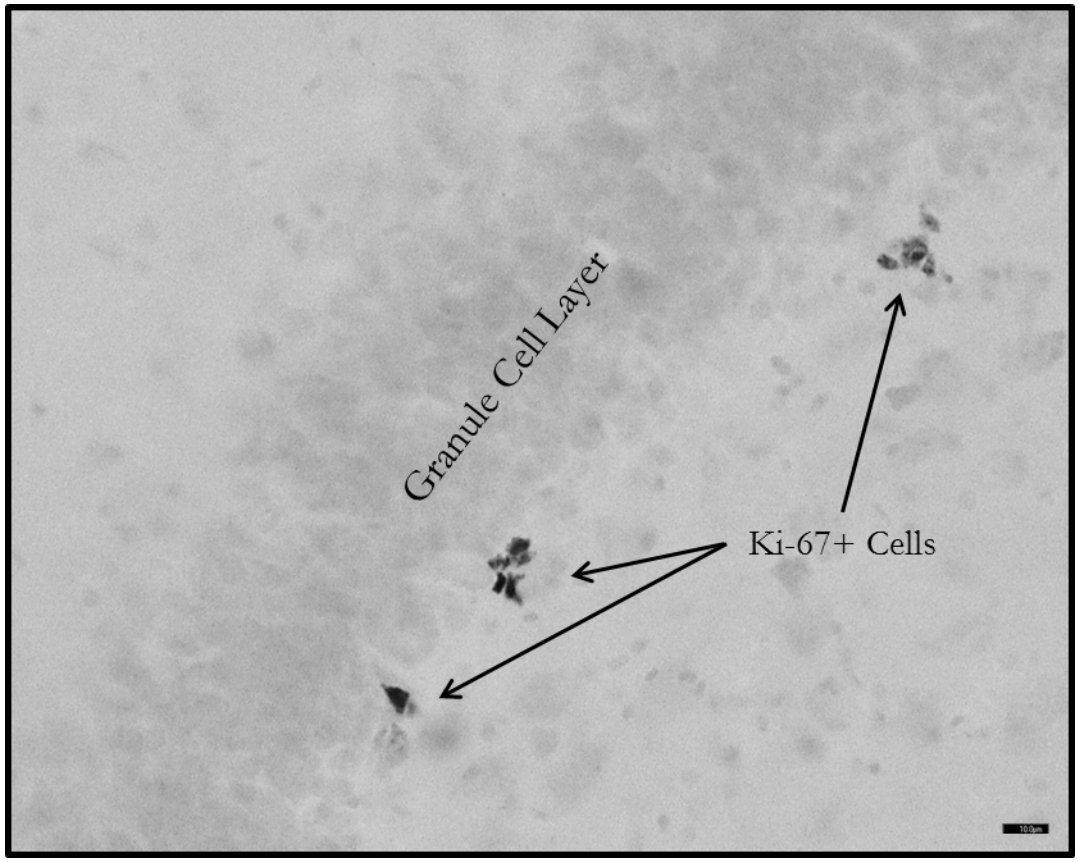

Figure 8.2. Representative Image of Ki-67+ Cells in the PD72 Rat Hippocampal Dentate Gyrus.

The cells are clustered together in the subgranular zone or inner edge of the granule cell layer. Image taken with a 40x lens, scale bar represents 10 µm.

8.2.4.2 Doublecortin (DCX) Immunohistochemistry

Doublecortin (DCX) was used to label immature dentate gyrus granule cells (Figure 8.3). The cells labeled were approximately 2-21 days old (Brown et al., 2003), were no longer actively dividing, and had committed to a neuronal fate. The most immature cells were located in the subgranular zone and had no visible dendrites, while older, more mature DCX+ cells had migrated farther into the granule cell layer and had at least one strong dendritic tree. Three sections of dorsal hippocampus ($1/16^{th}$) (~-3.1 to -4.3 mm DV bregma) were stained and used for analysis of the DCX population (n = 6-8/group). The protocol used:

1. Wash tissue in 0.1M Tris-buffered saline (TBS) 3 x 5 min
2. Incubation in 0.6% H2O2 in 0.1M TBS for 30 min
3. Wash tissue in 0.1M TBS 3 x 5 min
4. Incubation of sections in primary antibody (anti-DCX made in goat, Santa Cruz C-18, 1:250 dilution) made in blocking solution (3% normal donkey serum, 0.1% Triton-X in TBS) for 24 hours at 4°C
5. Wash tissue in 0.1M TBS for 3 x 5 min
6. Incubate sections in secondary antibody (donkey anti-goat biotinylated secondary, dilution 1:250, Vector) in blocking solution for one hour at room temperature
7. Wash tissue in 0.1M TBS 2 x 10 min
8. Wash tissue in blocking solution 1 x 10 min

9. Incubate tissue in Avidin-Biotin Complex solution (Vector) mixed in washing solution for one hour at room temperature
10. Wash tissue in 0.1M TBS 3 x 5 min
11. Visualize immunolabeling with 0.5 mg/ml nickel-enhanced diaminobenzedine (DAB) and 0.33 µl/ml H_2O_2 in 0.1M TBS
12. 0.1M TBS rinse for 1-2 sec
13. Wash tissue in 0.1M TBS 2 x 5 min

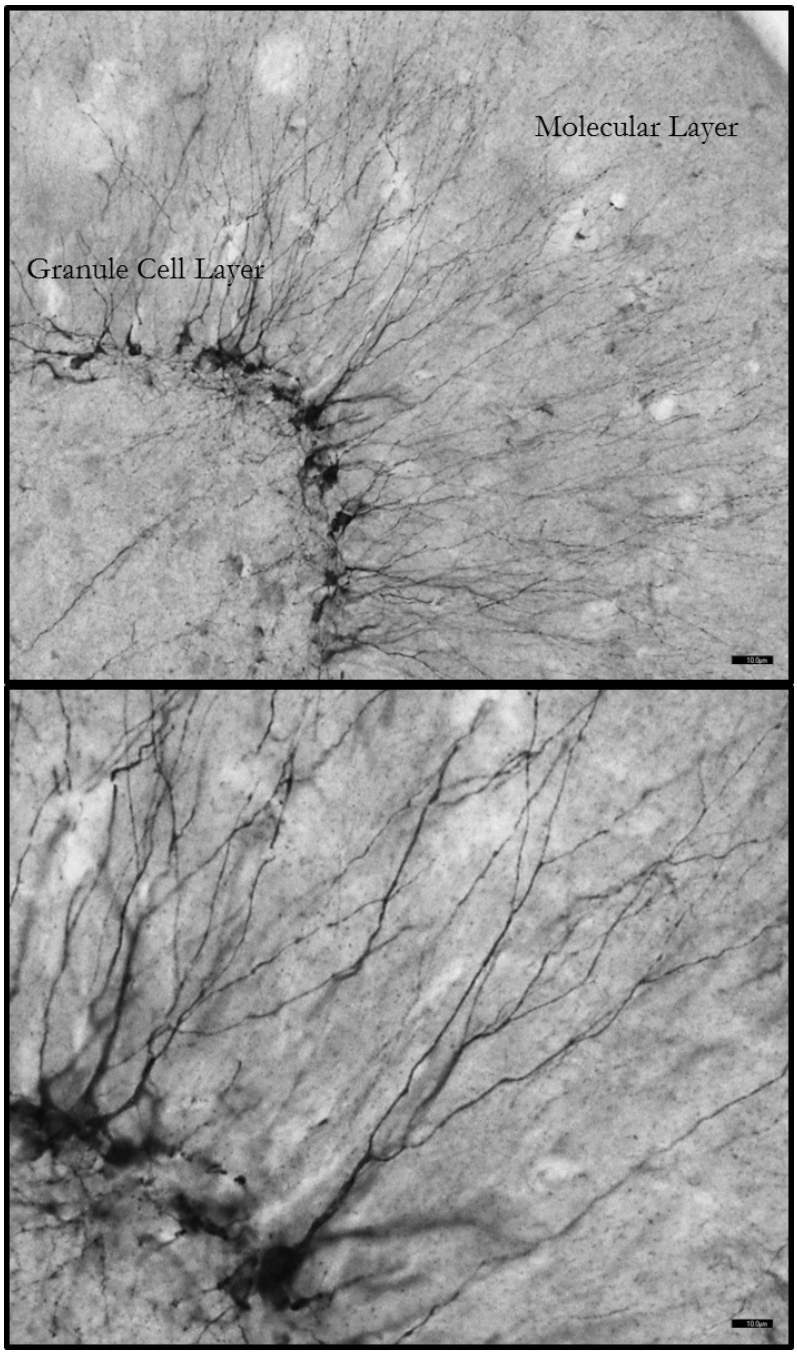

Figure 8.3. Representative Image of DCX+ Immature Neurons.

The cell bodies line the inside of the granule cell layer and the dendrites extend outward toward the molecular layer. Top image taken with a 20x lens and bottom image taken with a 40x lens, scale bars represent 10 µm.

8.2.5 Cell Count Estimates Using Unbiased Stereology

Cell quantification was performed in an unbiased stereological manner using the optical fractionator probe (Stereo Investigator, Micro Bright Field Inc., Williston, VT). For Ki-67, a grid and counting frame size of 200 µm x 200 µm was used. For DCX, a grid size of 200 µm x 200 µm and a counting frame size of 100 µm x 100 µm was used. In both cases, only the dorsal dentate gyrus was analyzed. For Ki-67, the section-sampling fraction was 1/8 and for DCX the fraction was 1/16. The section thickness fraction was the ratio of the dissector height (12 µm) to the mean thickness of the sections which was measured at every 5^{th} counting frame. Prior to counting the cells with a 40x lens, the experimenter drew a contour of the dentate gyrus (subgranular zone and granule cell layer) with a 5x lens. Slides were coded so that the experimenter was blind to treatment condition and counts were made within a known volume of the hippocampal region of interest. Only animals with a coefficient of error (CE) of less than 0.1 (m = 1) were included in analyses.

8.2.6 Dendritic Structure of Immature Neurons

Immature dentate gyrus granule cells (DCX+) were traced and Sholl analysis conducted using a computer-based neuron tracing system (NeuroLucida v. 10.52; MBF, Bioscience, Williston, VT). Two sections of dorsal dentate gyrus were used per animal for the analysis. Fully labeled DCX+ granule cells were identified and traced at 400x magnification (40x lens). Cells were in the analysis if they met criteria: 1) fully labeled DCX+ cell in postmitotic stage (presence of a dendrite extending towards the molecular layer (Plumpe et al., 2006)), 2) dendritic tree contained in the analyzed

section, and 3) the dendritic tree's branches were not broken or obscured. These criteria allowed for 6-10 cells to be traced per animal (n = 4-5 animals/group). Using the computer software to conduct a Sholl analysis, the number of intersections at each radius, total dendritic length per radius and number of nodes/bifurcations per radius was determined, which allowed for analysis of dendritic complexity. The average length of each cells' dendritic tree, average number of intersections, length per radius, and number of bifurcations per cell were also analyzed for each animal.

8.3 Results

8.3.1 Body Weights, BACs, and Running History

Repeated-measures ANOVAs were conducted to compare weights from PD4-9 and weights on PD30, 42, 50, 58, 64, and 72 were conducted. For PD4-9, a Day x Neonatal Condition interaction was found ($F_{(10,366)} = 5.206$, $p < 0.0001$), as well as main effects of Day ($F_{(5,366)} = 424.3$, $p < 0.0001$) and Neonatal Condition ($F_{(2,366)} = 85.47$, $p < 0.0001$) (Figure 8.4). Tukey's multiple comparison *post hoc* determined that on PD5-9, AE animals weighed less than SI and SC pups ($p < 0.01$ in all cases). For PD30-72, a Day x Housing interaction was found ($F_{(10,260)} = 3.971$, $p = 0.004$), as well as a main effect of Day ($F_{(5,260)} = 4947.221$, $p < 0.0001$). The Day x Neonatal interaction and the interaction between Day, Neonatal Condition, and Housing were not significant ($F < 1$). *Post hoc* tests (Tukey's HSD) revealed a significant decrease in weight for WRWR animals compared to the SH group ($p = 0.036$). WREC did not differ from either SH or WRWR animals.

BACs were analyzed from plasma collected on PD4 2 hours following the second alcohol exposure. The BACs averaged 334.4 mg/dl (±25.8 SEM) which is similar to previously published work from our lab (Hamilton et al., 2012; Boschen et al., 2014).

Running distance per 24 hours was measured per cage (3 animals/cage). For the first 12 days of wheel running access (PD31-42), average running distance was 2.86 miles (± 1.18 SD) per 24 hours. For WRWR animals, the average distance ran from PD43-72 was 9.2 miles (± 3.71 SD) per 24 hours. These running distances are consistent with previously published work from our lab using the WREC intervention (Hamilton et al., 2012; Boschen et al., 2014). The increase in running during the last 30 days in WRWR animals compared to the first two days could be due to the longer length/stride of the animals as they aged or increased natural running endurance.

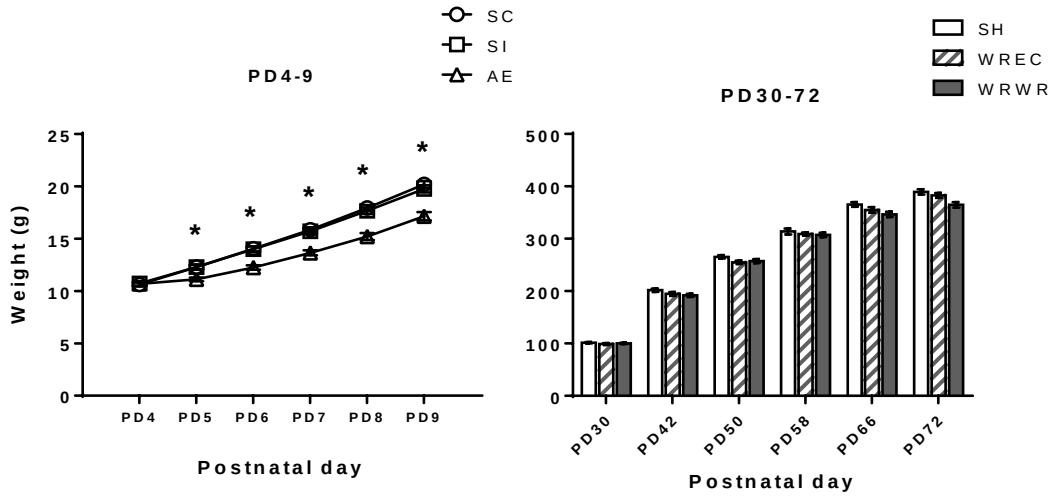

Figure 8.4. Postnatal Body Weights Across Conditions.

A) PD4-9 weights across the three neonatal conditions. By PD5, AE differed from SC and SI. B) PD30-72 weights by housing condition (collapsed across neonatal condition). * = $p < 0.05$. AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control; SH: Standard social housing; WREC: Wheel running followed by environmental complexity; WRWR: Wheel running.

8.3.2 Ki-67 Cell Counts

A two-way ANOVA was conducted to assess the number of proliferating (Ki-67+) cells in the PD72 dentate gyrus (Figure 8.5). Due to differences in labeling quality between batches, data were calculated as percent of control compared to the SC/SH group within each run. A significant main effect of housing was found ($F_{(2,30)} = 7.634$, $p = 0.0021$; Figure 8.4). Tukey's HSD test found that the WRWR group had significantly more Ki-67+ cells compared to the SH group ($p = 0.002$) and a trending increase compared to the WREC group ($p = 0.052$). WREC and SH groups did not differ in cell number. *Post hoc* tests (LSD) found a trend of a higher number of Ki-67+ cells in SC/WRWR rats compared to SC/SH and SC/WREC animals ($p = 0.08$ and

0.089, respectively). For SI, WRWR significantly increased the number of proliferating cells compared to the SH group ($p = 0.049$). WRWR did not differ from WREC. In the AE group, there were significantly more Ki-67 cells in both the WRWR and WREC groups compared to SH animals ($p = 0.013$ and 0.027, respectively). WREC and WRWR did not differ from one another.

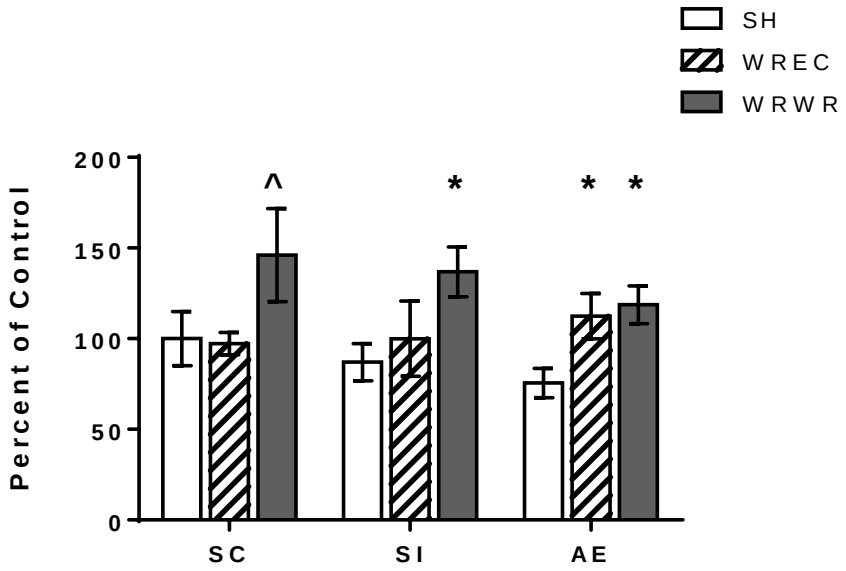

Figure 8.5. Continuous Wheel Running (WRWR) Increases the Number of Proliferating (Ki-67+) Cells in the PD72 Dorsal Hippocampal Dentate Gyrus.

For SC, more proliferation in the WRWR group vs. WREC and SH. For SI, more Ki-67+ cells WRWR vs. SH. For AE, more proliferation in WRWR and WREC vs. SH. * = $p < 0.05$, ^ = $p < 0.1$. AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control; SH: Standard social housing; WREC: Wheel running followed by environmental complexity; WRWR: Wheel running.

8.3.3 Doublecortin Cell Counts

Planned one-way ANOVAs were conducted to compare the number of DCX+ cells in the dentate gyrus of social-housed animals in each neonatal condition (Figure 8.6). A trending main effect of neonatal condition was found ($F_{(2,18)} = 3.054$, $p =$

0.072), with *post hoc* analysis (Tukey's) revealing a trending decrease in the number of DCX+ cells in the AE group compared to the SC group ($p = 0.066$). Cell counts in the SI group did not differ from the other conditions. Neonatal groups were then compared across the different housing conditions (SH vs. WREC vs. WRWR) to determine if the housing condition significantly altered the number of DCX+ cells in each neonatal treatment. For SC animals, a significant main effect of housing was found ($F_{(2,16)} = 5.257$, $p = 0.018$), and post hoc analysis found a significant increase in the number of DCX+ neurons in the WWR group compared to both the SH and WREC groups ($p = 0.033$ and $p = 0.029$, respectively). Cell number in the SC/SH and SC/WREC groups did not differ. For the SI group, a main effect of housing was observed ($F_{(2,17)} = 3.961$, $p = 0.039$). *Post hoc* analysis revealed a significant increase in the number of DCX+ cells in the WRWR group compared to the SH animals ($p = 0.03$). SI/WREC animals did not differ from SI/SH or SI/WRWR animals. For AE animals, a trending main effect of housing was found ($F_{(2,16)} = 2.919$, $p = 0.083$). Tukey's *post hoc* test found no significant differences between the housing conditions.

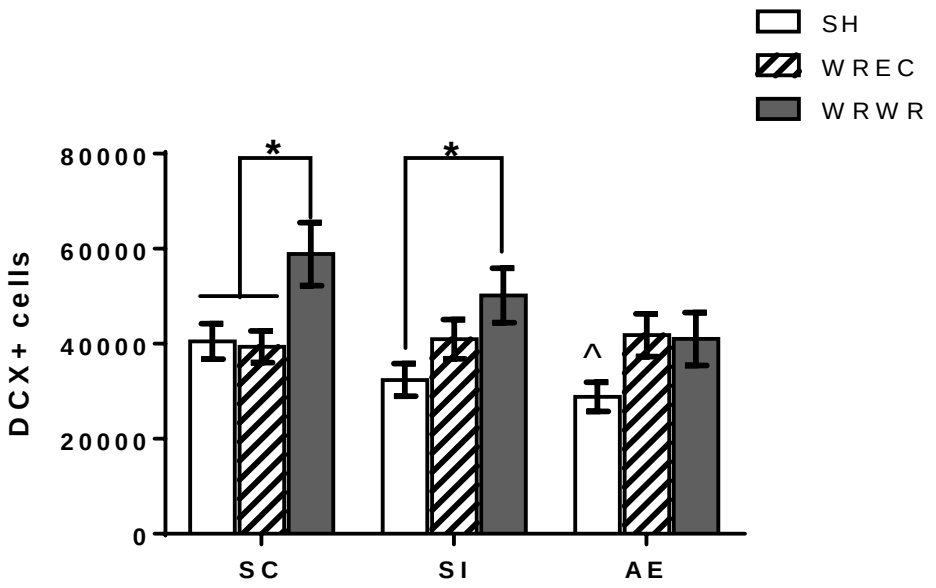

Figure 8.6. Continuous Wheel Running (WRWR) Increases the Number of Immature Neurons (DCX+) in the PD72 Dorsal Hippocampal Dentate Gyrus in Control Animals.

The number of DCX+ cells was increased in the SC/WRWR group compared to both SH and WREC. In the SI group, WRWR increased the number of DCX+ cells compared to the SH group. In AE animals, a trending decrease in the number of DCX+ cells was observed compared to the SC group ($p = 0.066$). * = $p < 0.05$, ^ = $p < 0.1$. AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control; SH: Standard social housing; WREC: Wheel running followed by environmental complexity; WRWR: Wheel running.

8.3.4 DCX+ Dendritic Morphology

Planned comparisons were conducted for each measure of dendritic complexity based on the Sholl analysis results. The three measures determined by the Sholl analysis were: amount of dendritic material (length) per radius, number of intersections per radius, and the number of dendritic bifurcations per radius. The planned repeated-measures ANOVAs compared: 1) AE/SH vs. SI/SH vs. SC/SH (Figure 8.7), 2) SC/SH vs. SC/WREC vs. SC/WRWR (Figure 8.9), 3) SI/SH vs. SI/WREC vs. SI/WRWR, and 4) AE/SH vs. AE/WREC vs. AE/WRWR (Figure 8.8). Representative tracings are shown in Figure 8.7-8.9 and statistical output is summarized in Table 1.

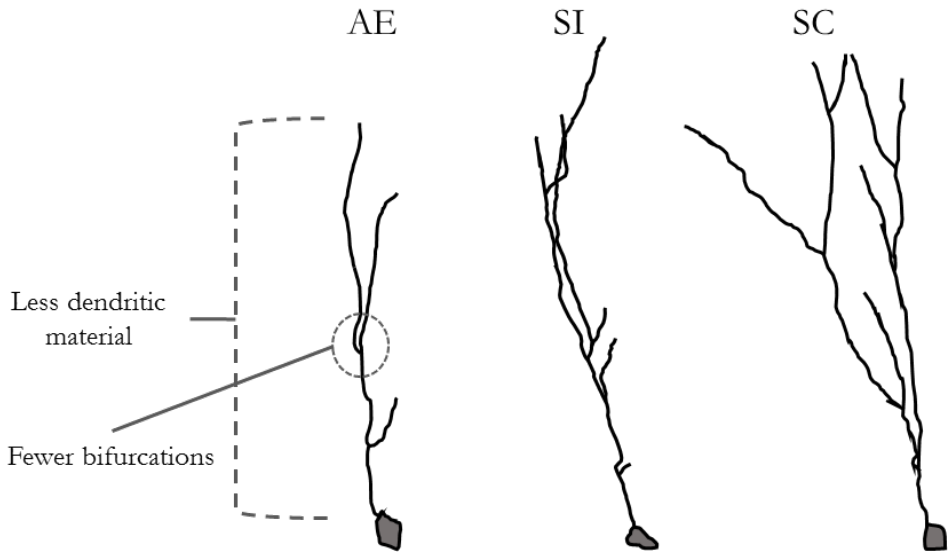

Figure 8.7. Representative Neurolucida Tracings of DCX+ Immature Neurons in the PD72 Dorsal Hippocampal Dentate Gyrus.

All tracings are of neurons from animals housed in standard social housing (SH) to assess differences in baseline dendritic morphology between neonatal conditions. AE: Alcohol-exposed, SI: Sham-intubated, SC: Suckle control. Reproduced with permission from Boschen & Klintsova, *in press*.

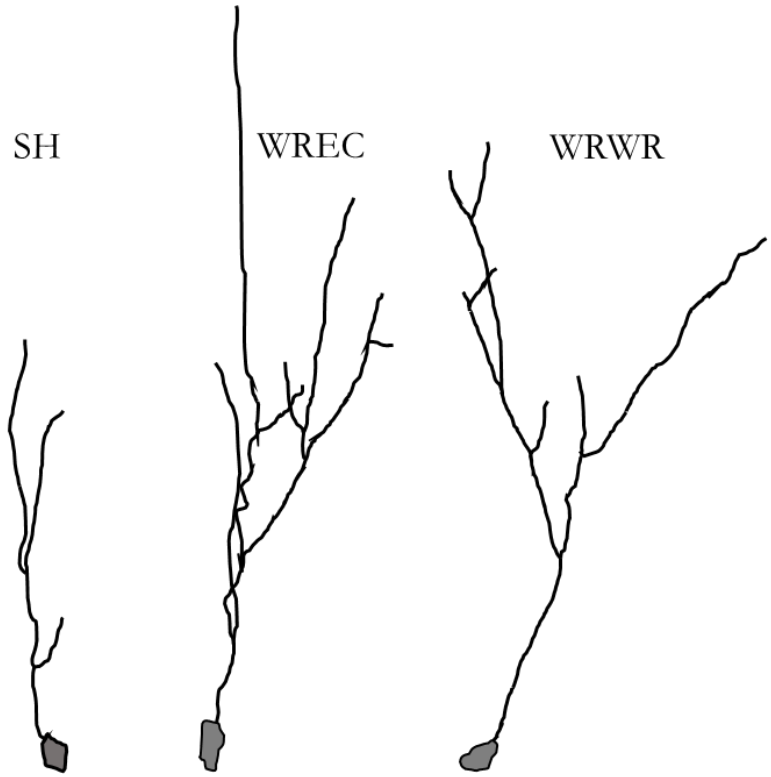

Figure 8.8. Representative NeuroLucida tracings of DCX+ immature neurons in the alcohol-exposed (AE) PD72 dorsal dentate gyrus.

WREC and WRWR significantly increased the amount of dendritic material, intersections, and bifurcations. SH: Standard social housing; WREC: Wheel running followed by environmental complexity; WRWR: Wheel running.

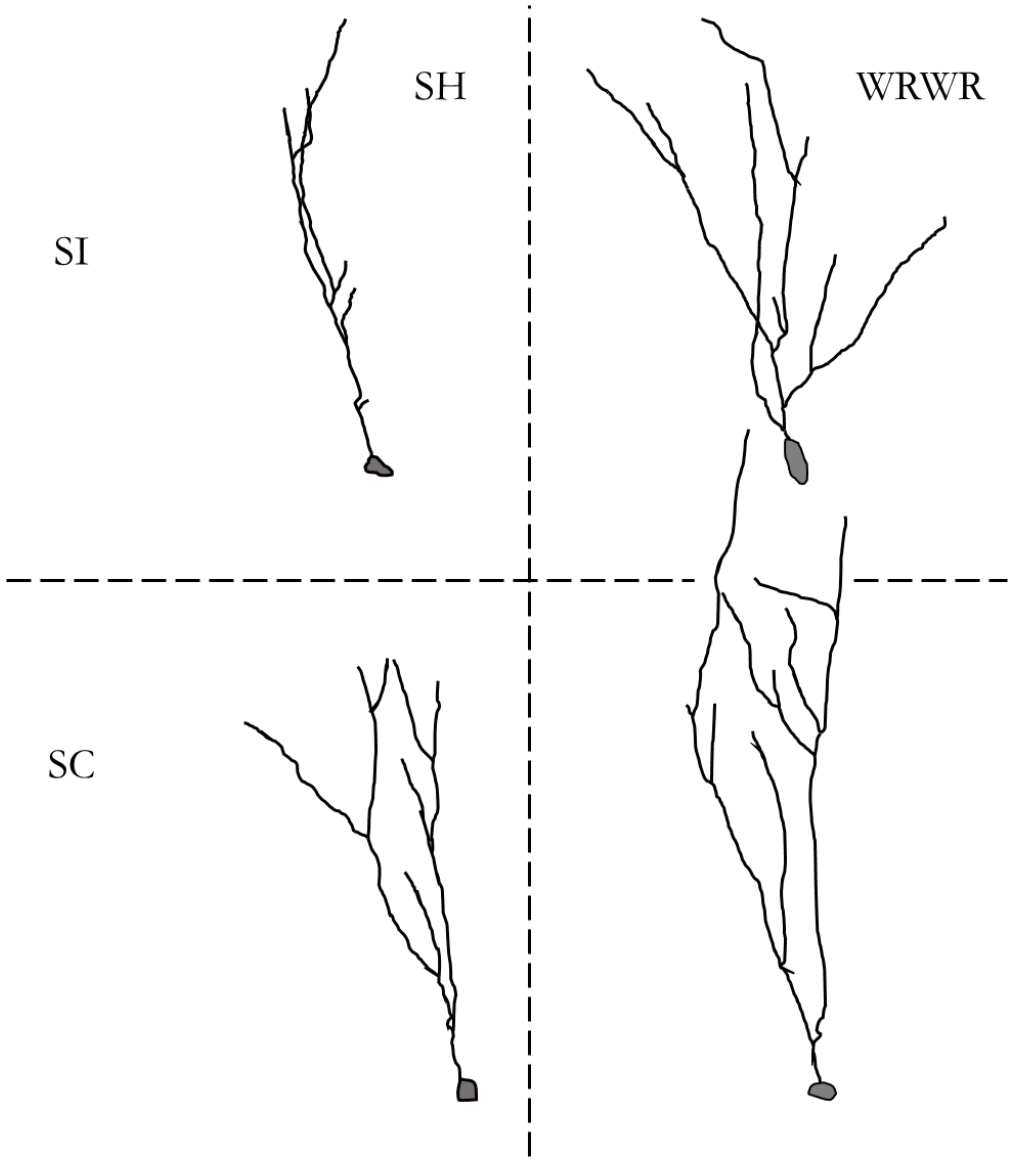

Figure 8.9. Representative NeuroLucida tracings of DCX+ immature neurons in control animals.

WRWR increased the number of bifurcations and amount of dendritic material compared to dendrites in SH animals. WREC (not shown) did not significantly affect these measures in control animals. SI: Sham-intubated; SC: Suckle control; SH: Standard social housing; WREC: Wheel running followed by environmental complexity; WRWR: Wheel running.

8.3.4.1 Length per Radius

For the comparison of neonatal treatments housed in the SH condition, a radius x neonatal condition interaction was found ($F_{(26,156)} = 3.671$, $p < 0.0001$; Figure 8.9A). Main effects were also found for radius ($F_{(13,156)} = 184.8$, $p < 0.0001$) and for neonatal condition separately ($F_{(2,12)} = 7.851$, $p = 0.0066$). *Post hoc* analysis (Tukey's multiple comparison test) revealed a significant decrease in length for AE compared to SI for radii 90 and 110 µm from the soma ($p < 0.0001$ for both), significant decreases for AE compared to SC for radii 50, 70, 90, 110 and 130 µm from the soma ($p < 0.001$, 0.0001, 0.0001, 0.001 and 0.01, respectively) and significant decreases in SI compared to SC for radii 50 and 70 µm ($p < 0.01$ for both radii).

Next, we compared the housing conditions for each neonatal treatment (Figure 8.9B-D). For SC animals, a main effect of radius ($F_{(13,140)} = 101.7$, $p < 0.0001$; Figure 8.9D) and housing were found ($F_{(2,140)} = 6.393$, $p = 0.0022$), but the interaction was not significant ($F < 1$). *Post hoc* analysis (Tukey's) found a significant increase in dendritic material between SH and WRWR only at radius 90 µm from the soma. For the SI group, a main effect of radius ($F_{(13,140)} = 132.5$, $p < 0.0001$) and housing condition were observed ($F_{(2,140)} = 16.19$, $p < 0.0001$), but no significant interaction was found ($F_{(26,140)} = 1.328$, $p = 0.1502$; Figure 8.9C). *Post hoc* tests revealed a significant increase in length per radius in the WRWR condition compared to both SH and WREC at radii 50 and 70 µm ($p < 0.05$ in all cases) and for WRWR compared to only SH at radius 90 µm from the soma ($p < 0.01$). For AE animals, a significant radius x housing interaction was found ($F_{(26,140)} = 2.859$, $p < 0.0001$; Figure 8.9B), as

well as main effects for radius ($F_{(13,140)} = 122.8$, $p < 0.0001$) and housing ($F_{(2,140)} = 13.05$, $p < 0.0001$). Tukey's *post hoc* analysis found significantly increased amounts of dendritic material per radius in WRWR and WREC conditions compared to SH for radii 70, 90, 110, and 130 µm from the some ($p < 0.01$ at 70 µm, $p < 0.0001$ for 90 um, $p < 0.001$ for 110 µm, and $p < 0.05$ um for 130 µm).

Measure	Planned Comparison	Interaction	Radius	Neonatal Treatment	Housing Condition	Significant *post hocs* (µm)
Dendritic Material	SH: AE vs. SI vs. SC	****	****	**	N/A	AE vs. SI: 90, 110 AE vs. SC: 50, 70, 90, 110, 130 SI vs. SC: 50, 70
	SC: SH vs. WREC vs. WRWR	n.s.	****	N/A	**	SH vs. WRWR: 90
	SI: SH vs. WREC vs. WRWR	n.s.	****	N/A	****	SH vs. WREC: 50, 70 SH vs. WRWR: 90 WREC vs. WRWR: 50, 70
	AE: SH vs. WREC vs. WRWR	****	****	N/A	****	SH vs. WREC: 70, 90, 110, 130 SH vs. WRWR: 70, 90, 110, 130
Intersections per Radius	SH: AE vs. SI vs. SC	****	****	**	N/A	AE vs. SI: 70, 90, 110 AE vs. SC: 30, 50, 70, 90, 110, 130 SI vs. SC: 50, 130
	SC: SH vs. WREC vs. WRWR	n.s.	****	N/A	*	SH vs. WRWR: 90
	SI: SH vs. WREC vs. WRWR	*	****	N/A	****	SH vs. WREC: 30, 50, 70 WREC vs. WRWR: 30, 50, 70
	AE: SH vs. WREC vs. WRWR	***	****	N/A	****	SH vs. WREC: 50, 70, 90, 110 SH vs. WRWR: 50, 70, 90, 110
Bifurcations per Radius	SH: AE vs. SI vs. SC	n.s.	****	**	N/A	AE vs. SI: 50, 70 AE vs. SC: 30, 50
	SC: SH vs. WREC vs. WRWR	n.s.	****	N/A	n.s.^	SH vs. WREC: 110 SH vs. WRWR: 90
	SI: SH vs. WREC vs. WRWR	n.s.	****	N/A	n.s.^	SH vs. WREC: 110 SH vs. WRWR: 90
	AE: SH vs. WREC vs. WRWR	n.s.	****	N/A	****	SH vs. WREC: 70, 90, 110 SH vs. WRWR: 50

Table 8.1. Summary of Statistical Output for Dendritic Complexity Analyses.

***Post hocs* refer to radii (µm from the soma) where significant differences were found ($p < 0.05$). * = $p < 0.05$, ** $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n.s. = not significant, ^ = $p < 0.01$. AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control; SH: Standard social housing; WREC: Wheel running followed by environmental complexity; WRWR: Wheel running.**

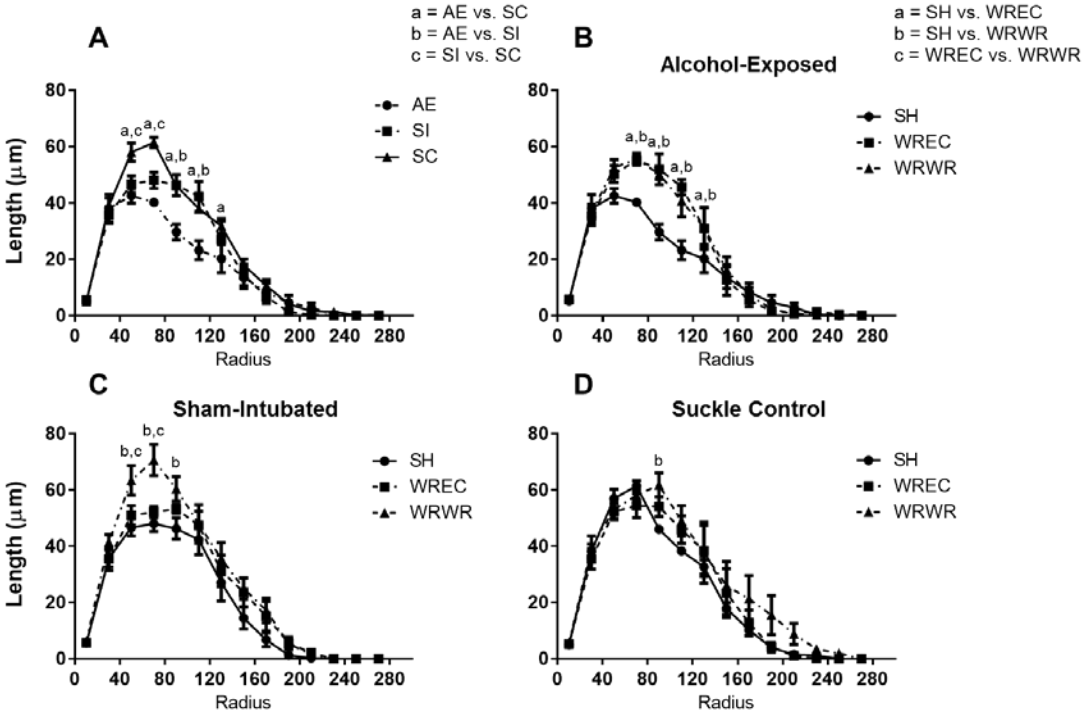

Figure 8.10. WRWR and WREC Rescue Alcohol-Induced Alterations to Amount of Dendritic Material per Radius in the PD72 Dorsal Dentate Gyrus.

A) AE significantly decreased amount of dendritic material per radius in immature dentate granule cells. For this graph, significant differences ($p < 0.05$) at each radii are indicated as a = AE vs. SC, b = AE vs. SI, and c = SI vs. SC. B) In AE animals, WRWR and WREC ameliorated the negative impact of neonatal alcohol exposure on measures of dendritic material. In graphs B-D, significant differences between housing conditions are indicated as a = SH vs. WREC, b = SH vs. WRWR, and c = WREC vs. WRWR. C and D) In control animals, WRWR increased dendritic material at certain radii. For SC, WRWR increased dendritic material compared to SH only at 90 μm from the soma. Values indicate means ± SEM. AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control; SH: Standard social housing; WREC: Wheel running followed by environmental complexity; WRWR: Wheel running.

8.3.4.2 Number of Intersections per Radius

For the comparison of neonatal treatments housed in the SH condition on number of intersections, a radius x neonatal condition interaction was found ($F_{(26,156)}$ = 3.194, $p < 0.0001$; Figure 8.10A). Main effects were also found for radius ($F_{(13,156)}$ = 150.4, $p < 0.0001$) and for neonatal condition separately ($F_{(2,12)} = 9.42$, $p = 0.0035$). *Post hoc* analysis (Tukey's) revealed a significant decrease in number of intersections for AE compared to SI for radii 70, 90, and 110 µm from the soma ($p < 0.05$ for all), significant decreases for AE compared to SC for radii 30, 50, 70, 90, 110 and 130 µm from the soma ($p < 0.05$ in all cases) and significant decreases in SI compared to SC for radii 50 and 130 µm ($p < 0.01$ and $p < 0.05$, respectively).

We then compared the housing conditions for each neonatal treatment (8.10B-D). For SC animals, a significant main effect of radius ($F_{(13,140)} = 75.6$, $p < 0.0001$) and of housing were found ($F_{(2,140)} = 3.607$, $p = 0.0297$), but the interaction was not significant ($F < 1$; Figure 8.10D). *Post hoc* tests found a significant increase in number of intersections per radius in WRWR compared to SH animals at radius 90 µm from the soma ($p < 0.05$). For the SI group, a radius x housing interaction ($F_{(26,140)} = 1.706$, $p = 0.0263$; Figure 8.10C), a main effect of radius ($F_{(13,140)} = 136.1$, $p < 0.0001$), and a main effect of housing condition were observed ($F_{(2,140)} = 17.36$, $p < 0.0001$). *Post hoc* tests revealed a significant increase in intersections per radius for WRWR animals compared to both SH and WREC at radii 30, 50 and 70 µm ($p < 0.05$ for 30 µm, $p < 0.001$ for 70 µm, and $p < 0.01$ for 90 µm). For AE animals, a significant radius x housing interaction was found ($F_{(26,140)} = 2.488$, $p = 0.0004$; Figure 8.10B), as well as

main effects for radius ($F_{(13,140)} = 109.2$, $p < 0.0001$) and housing ($F_{(2,140)} = 13.42$, $p < 0.0001$). *Post hoc* analysis found significantly increased number of intersections per radius in WRWR and WREC conditions compared to SH for radii 50, 70, 90, and 110 µm from the soma ($p < 0.05$ at 50 µm, $p < 0.01$ at 70 µm, $p < 0.001$ for 90 µm, $p < 0.05$ for 110 µm). WREC and WRWR did not differ from one another.

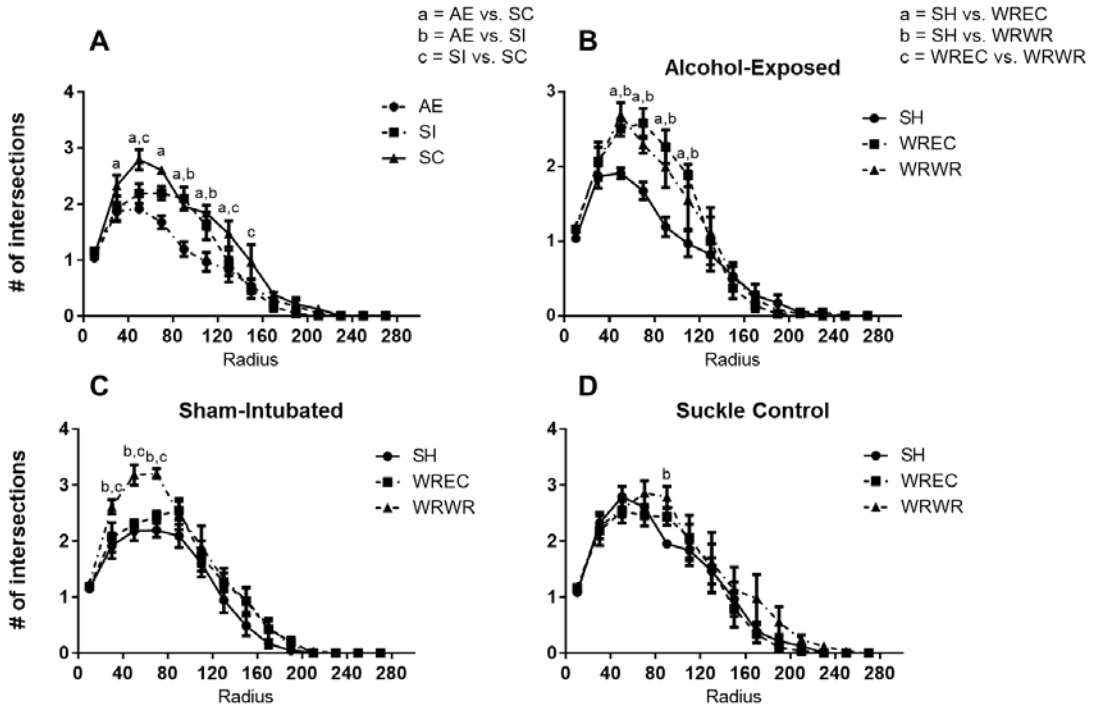

Figure 8.11. WRWR and WREC Rescue Alcohol-Induced Decreases in Intersections per Radius in the PD72 Dorsal Dentate Gyrus.

A) AE significantly decreased the number of intersections per radius in immature dentate granule cells ($p < 0.05$). B) In AE animals, WRWR and WREC mitigated the negative impact of neonatal alcohol exposure on number of intersections. C and D) In control animals, WRWR increased the number of intersections compared to SH and WREC for SI animals. For SC, WRWR increased intersections compared to SH only at 90 µm from the soma. Values indicate means ± SEM. AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control; SH: Standard social housing; WREC: Wheel running followed by environmental complexity; WRWR: Wheel running.

8.3.4.3 Number of Bifurcations per Radius

We first compared neonatal treatments housed in the SH condition on number of intersections and found a main effect of radius ($F_{(13,156)} = 46.46$, $p < 0.0001$; Figure 8.11A) and of neonatal condition ($F_{(2,12)} = 9.976$, $p = 0.0028$), but no significant interaction was revealed. *Post hoc* tests (Tukey's) found a significant decrease in number of bifurcations per radius for AE compared to SI for radii 50 and 70 µm from the soma ($p < 0.001$ and $p < 0.01$, respectively) and significant decreases for AE compared to SC for radii 30 and 50 µm from the soma ($p < 0.01$ and $p < 0.05$, respectively).

We then compared the housing conditions for each neonatal treatment (Figure 8.11B-D). For SC animals, a significant main effect of radius ($F_{(13,130)} = 39.25$, $p < 0.0001$) and a trending main effect of housing were found ($F_{(2,10)} = 3.737$, $p = 0.0614$), but the interaction was not significant ($F_{(26,130)} = 1.226$, $p = 0.2268$; Figure 8.11D). *Post hoc* analysis (Tukey's) found a significant increase in number of intersections per radius in WRWR compared to SH animals at radius 90 µm from the soma ($p < 0.001$) and in WREC compared to SH at radius 110 µm ($p < 0.05$). For the SI group, a main effect of radius ($F_{(13,140)} = 46.33$, $p < 0.0001$; Figure 8.11C) and a trending main effect of housing were observed ($F_{(2,10)} = 4.052$, $p = 0.0514$). *Post hoc* tests revealed a significant increase in intersections per radius for WRWR animals compared to both SH and WREC at radius 50 µm ($p < 0.001$ and 0.05, respectively) and an increase in WRWR compared to SH alone at radius 30 µm ($p < 0.001$). For AE animals, no interaction was found, but main effects for radius ($F_{(13,130)} = 41.44$, $p < 0.0001$) and

housing ($F_{(2,130)} = 30.88$, $p < 0.0001$) were revealed (Figure 8.11B). *Post hoc* tests found significantly increased number of bifurcations per radius in WRWR compared to SH for radii 50 and 90 µm from the soma ($p < 0.001$ at 50 um and $p < 0.05$ for 90 um) and increased in WREC compared to SH for radii 70, 90, and 110 µm from the soma ($p < 0.01$, 0.01, and 0.05 respectively). WREC and WRWR did not differ.

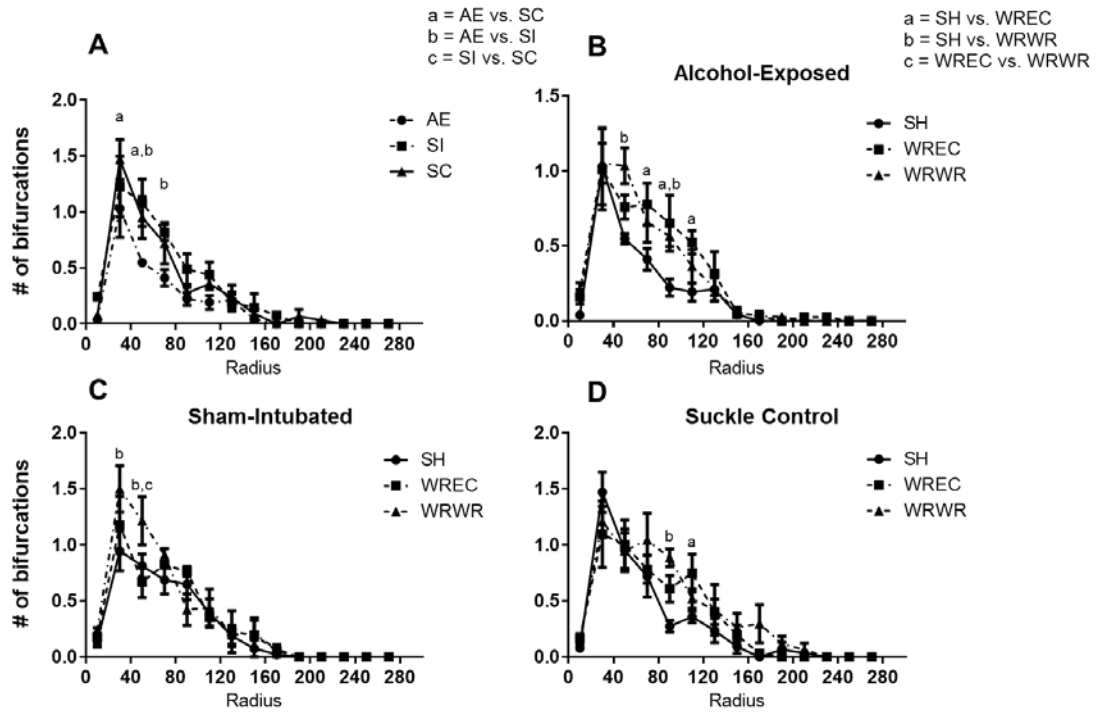

Figure 8.12. WRWR and WREC Rescue Alcohol-Induced Decreases in Number of Bifurcations per Radius in the PD72 Dorsal Dentate Gyrus.

A) AE significantly decreased the number of bifurcations per radius in immature dentate granule cells ($p < 0.05$). B) In AE animals, WRWR and WREC reversed the negative impact of neonatal alcohol exposure on number of intersections. C and D) In control animals, WRWR also increased the number of bifurcations, though only at 30 and 50 µm radii in SI and at 90 µm in SC. For SC, WREC increased number of bifurcations at 70 µm as well. Values indicate means ± SEM. AE: Alcohol-exposed; SI: Sham-intubated; SC: Suckle control; SH: Standard social housing; WREC: Wheel running followed by environmental complexity; WRWR: Wheel running.

8.4 Discussion

Specific Aim 1 demonstrated that neonatal alcohol exposure (PD4-9) decreased dendritic complexity of immature (DCX+) neurons in the rat dorsal dentate gyrus while not affecting the number of immature neurons or new cell proliferation on PD72. In addition, continuous wheel running (WRWR) and wheel running followed by environmental complexity (WREC) both reversed alcohol-related dendritic abnormalities. However, these interventions had differential effects on cell proliferation and the number of immature neurons in control animals. Specifically, WRWR enhanced the number of proliferating cells and immature neurons, while WREC had no effect on these measures. In addition, WRWR increased dendritic complexity in SI and SC animals while WREC caused few to no alterations to dendritic morphology. Overall, these findings suggest that both WREC and WRWR are robust interventions for the reduced dendritic complexity observed in alcohol-exposed (AE) animals.

Cell proliferation was measured using the marker Ki-67, a marker present during and necessary for all active stages of the cell cycle. No effect of neonatal condition was observed which replicates previous work from our lab using this model of FASD (Klintsova et al., 2007; Hamilton et al., 2012, 2014). WRWR increased the number of Ki-67+ cells in all neonatal conditions, which is consistent with literature showing that exercise upregulates cell proliferation processes (van Praag, 2009; Vivar et al., 2013; Eadie et al., 2005). Furthermore, WREC did not alter proliferation in the control groups but did significantly increase the number of Ki-67+ cells in the AE

group. Previous experiments from our lab using similar time points of tissue analysis have not reported changes to proliferation following WREC (Hamilton et al., 2011, 2012, 2014) and, in general, it is thought that WREC enhances cell survival as opposed to proliferation (Olson et al., 2006). However, the effect of WREC and WRWR was similar in AE animals across all of the measures analyzed in this experiment (DCX+ cell counts and dendritic morphology), suggesting that for this particular experiment WREC was just as effective as WRWR alone in the alcohol-damaged brain. This Aim also focused on dorsal dentate gyrus only, which was not the case in our previous work. It is possible that the effect of WREC is more robust in the dorsal hippocampus and when dorsal and ventral areas are counted together the effect is washed out.

Similar results were observed following assessment of the number of DCX+ cells. DCX labels a heterogeneous population of neural progenitors and immature neurons, with expression peaking 7-14 days after proliferation (Brown et al., 2003). Neonatal condition did not affect the number of DCX+ cells, though there was a trending decrease between AE and SC ($p = 0.066$). Previously published work from our research group (Hamilton et al., 2011) did not find alcohol-related differences in DCX+ cell number; however, this study analyzed the entire dentate gyrus while the current experiments focused on the dorsal region only. Ieraci and Herrera (2007) found decreased cell proliferation in dorsal but not ventral dentate gyrus following a single binge of alcohol on PD7, supporting the differential effect of alcohol along the dorsoventral axis. In addition, DCX+ cells represent a population which widely varies

in cell age, from a few days to three weeks old. It is possible that the trending decrease observed in AE animals is indicative of fewer cells within one of these subpopulations. Specifically, the decreases in dendritic complexity in DCX+ cells reported in this dissertation combined with the fact that our lab has previously observed decreased cell survival 30 days following a BrdU injection (exogenous marker which labels proliferating cells) (Klintsova et al., 2007; Hamilton et al., 2011, 2012, 2014) suggests that the older population of DCX+ cells might be more vulnerable to the damaging effects of alcohol. Further research focused on this older subpopulation of DCX+ neurons is necessary.

WRWR, but not WREC, robustly increased the number of immature neurons in the control animals. This finding is in line with literature reporting increased DCX+ cell number following exercise (Kronenberg et al., 2006; Van der Borght et al., 2007, 2009). Interestingly, neither WRWR nor WREC significantly affected DCX+ cells in the AE group, suggesting that exercise was not as effective in these animals. This finding is in contrast to an earlier study from the Klintsova lab reporting increased number of DCX+ cells in AE animals following 12 days of WR and no exercise-induced alterations to DCX+ cells in control animals (Helfer et al., 2007). The exact reasons for these discrepant findings is not known, though the 2007 paper assessed adult neurogenesis following 12 days of exercise vs. 42 days in the current study and tissue was harvested at PD42 vs. PD72. Visual inspection of the data suggests a small increase in DCX+ cell number in both the AE/WRWR and AE/WREC conditions compared to SH, making it possible that the lack of significant effect was because of

low statistical power due to a relatively low sample size. WRWR did increase cell proliferation and dendritic complexity in AE animals which shows that adult neurogenesis in this group benefits from exercise.

Dendritic morphology of immature neurons within the dorsal dentate gyrus was negatively impacted by neonatal alcohol exposure. Sholl analysis revealed significantly simpler dendrites in AE animals compared to SI and SC on the measures of total dendritic material, number of intersections, and bifurcations. These results are consistent with previous work from our lab showing abnormalities in basilar pyramidal cell dendrites in Layer II/III of the medial prefrontal cortex following our model of FASD (Hamilton et al., 2010, 2015). Prenatal models of alcohol exposure also disrupt dendritic organization in the dorsal hippocampus (Sakata-Haga et al., 2003). These findings directly relate to the impaired adult-born granule cell survival following neonatal alcohol exposure (Klintsova et al., 2007; Hamilton et al., 2011, 2012, 2014). As discussed above, newly generated neurons undergo a process known as “competitive survival” following the initiation of glutamatergic input from the perforant pathway (Kempermann, 2011). An insufficient number of synaptic connections would impair a cell’s ability to successfully integrate during this process and decrease the number of surviving neurons. These data reported here suggest that the subpopulation of immature neurons about to enter competitive survival and receive glutamatergic input have less complex dendrites which would impair the ability of these cells to make synapses with the hippocampal circuit. Thus, the dendritic abnormalities in AE animals demonstrated here could represent a mechanism through

which neonatal alcohol exposure decreases granule cell survival. In addition, increased dendritic complexity following housing in the behavioral interventions could be an avenue through which WRWR and WREC rescue cell survival.

Both WRWR and WREC dramatically enhanced dendritic complexity of DCX+ immature neurons in AE animals and reversed alcohol-induced deficits. The amount of dendritic material and the number of intersections and bifurcations per radius were all increased following housing in either intervention. Exercise has repeatedly been shown to increase dendritic length and complexity in dentate gyrus granule cells (Eadie et al., 2005; Redila & Christie, 2006; Stranahan et al., 2007). Housing in a complex environment also alters dendritic morphology in hippocampal dentate gyrus and CA1 (Faherty et al., 2003) and other brain regions, such as the cortex and striatum (Faherty et al., 2003; Gelfo et al., 2009). More complex dendritic trees likely contribute to behavioral and cognitive enhancement observed following exercise and EC (Marlatt et al., 2012; Vaynman et al., 2004; Kohman et al., 2011; Green et al., 2011; Hamilton et al., 2011; Schreiber et al., 2013). Interestingly, while WRWR modestly enhanced dendritic complexity in the SI and SC group, WREC had almost no effect on these measures (apart from at one radius for number of bifurcations in SC animals). Additionally, the exercise-induced alterations were not as robust in the controls animals as in the AE group, suggesting a ceiling of plasticity in the healthy brain. The lack of effect of WREC on any measure of adult neurogenesis or dendritic complexity in the control animals indicates that the alcohol-exposed brain is more susceptible to benefit from this behavioral intervention.

BDNF is critical for dendritic outgrowth and adult neurogenesis (McAllister et al., 1997; Tolwani et al., 2002; Alonso et al., 2002; Sairanen et al., 2005), and neurotrophins are upregulated by both EC and exercise (Angelucci et al., 2009; Cotman & Berchtold, 2002; Rasmussen et al., 2009; Torasdotter et al., 1996, 1998). Thus, BDNF makes a likely candidate as a mechanism for 1) the alcohol-induced decreases in dendritic complexity in DCX+ neurons, and 2) the upregulation of cell proliferation and increased dendritic complexity observed in the current experiment. Interestingly, the decreases in dendritic complexity in the AE group occur independently of hippocampus-wide changes to BDNF levels (Specific Aim 2). Additionally, the alterations to dendritic morphology seen in the AE group following WRWR and WREC occur despite no change in hippocampus-wide BDNF levels in the AE/WREC condition. Furthermore, WRWR increases BDNF levels in the SC and SI groups while causing only modest alterations to dendritic complexity. These discrepancies make a direct comparison between the findings in Specific Aims 2 and 3 ill-advised. As discussed in Specific Aim 2, BDNF levels were analyzed from whole hippocampal tissue, including dorsal and ventral regions, while adult neurogenesis and dendritic complexity were only analyzed in dorsal dentate gyrus. Additionally, DCX labels only a subpopulation of immature neurons and dendritic complexity was measured in, essentially, the oldest subpopulation of this subpopulation. Thus, BDNF could still contribute to the results reported here, but a direct relationship cannot be determined from these experiments.

8.5 Conclusions

In summary, the experiment described in Specific Aim 3 found that neonatal (PD4-9) alcohol exposure negatively impacted dendritic complexity of immature neurons in the dorsal dentate gyrus while not affecting new cell proliferation. Furthermore, WREC and WRWR robustly enhanced dendritic complexity and cell proliferation in the alcohol-exposed brain. WRWR increased proliferation and dendritic complexity in the SC and SI groups while WREC had no effect on these measures. The results reported here suggest that our model of FASD targets maturing neurons prior to receiving excitatory synaptic input necessary for their long-term survival, possibly disrupting the ability of these neurons to make sufficient number of functional synapses. Impairments to the process of adult neurogenesis likely contribute to hippocampal-associated behavioral deficits observed in animal models of FASD. Importantly, these results also support WREC and WRWR as behavioral therapies in models of FASD as both are beneficial to neuroplasticity in the alcohol-damaged brain.

Chapter 9

SUMMARY AND CONCLUSIONS

Alcohol exposure during the third trimester-equivalent has a devastating impact on neuroplastic processes in late-developing structures such as the hippocampus and prefrontal cortex. Work from our laboratory and in collaborations with others has shown that our model of FASD induces apoptosis in CA1 with 24 hours of exposure (Gursky et al., *in preparation*), impairs hippocampal adult neurogenesis (Klintsova et al., 2007; Hamilton et al., 2011, 2012), decreases dendritic complexity and spine density of pyramidal cells in medial prefrontal cortex (Hamilton et al., 2010, 2015; Whitcher et al., 2008), downregulates expression of neuronal activity markers (Murawski et al., 2012), and disrupts performance on a variety of cerebellar- and hippocampal-associated tasks (Klintsova et al., 2002; Schreiber et al., 2013; Hamilton et al., 2014). Based on this catalog of evidence, the first goal of this dissertation was to explore neurotrophins, in particular BDNF, as a molecular mechanism which might be altered by neonatal alcohol exposure and be linked to decreased neuroplasticity in the alcohol-exposed infant and adult rat hippocampus.

In Specific Aim 1, I first asked: *does neonatal alcohol exposure (PD4-9) alter BDNF and TrkB protein expression, Bdnf gene expression, and Bdnf DNA methylation in the neonatal hippocampus?* Aim 1, Experiment 1 addressed this question and found that our model of FASD (AE) increases BDNF and TrkB protein levels, total and exon-specific *Bdnf* gene expression, and was associated with less *Bdnf* Exon I

methylation. This experiment also reported some overlapping effects of sham-intubation (SI) on BDNF and increased plasma corticosterone levels in AE and SI pups, giving us valuable information regarding the role of stress in our model and the importance of developing new techniques to minimize stress during the alcohol exposure procedure. Overall, the upregulation of BDNF synthesis could indicate a compensatory neuroprotective response of the brain to insult.

I also sought to investigate the neuroimmune response as a secondary source of alcohol-induced damage in the neonatal hippocampus. Thus, the second question asked in Aim 1 was: *does neonatal alcohol exposure impact microglial cell number and morphology or the production of pro- and anti-inflammatory cytokines in the neonatal hippocampus?* Our lab's model of FASD decreased the number of microglia in CA1 and the dentate gyrus, while the remaining microglia were more activated and exhibited increased pro- and anti-inflammatory gene expression. Microglial number was also decreased in the SI group and there was an upregulation of pro-inflammatory cytokine expression in these animals, again supporting the further investigation of stress in our alcohol exposure model. The increased production of pro-inflammatory cytokines following AE could further exacerbate alcohol-related damage and prime the immune system to overactivate following a second immune challenge in adulthood. Interestingly, levels of the anti-inflammatory cytokine were only upregulated in AE pups, again suggesting a possible neuroprotective response to alcohol-induced insult.

Most of our laboratory's work has focused on the adult hippocampus and reported significant disruptions to neuroplasticity (Klintsova et al., 2007; Hamilton et al., 2012; Murawski et al., 2012). To extend the work done in Aim 1 and to determine if alterations to BDNF could be contributing to deficits in hippocampal neuroplasticity, for Specific Aim 2 I asked: *does neonatal alcohol exposure (PD4-9) alter Bdnf exon-specific gene expression, BDNF protein, or DNA methylation in the adult rat hippocampus?* In this experiment, I reported that third trimester-equivalent alcohol exposure did not alter basal expression of BDNF protein, total or exon-specific mRNA levels, or exon I DNA methylation. These findings suggest that the increased levels of BDNF reported in Aim 1 are transient and do not persist into adulthood.

Previous work from our lab has explored the use of wheel running (WR) and housing in a complex environment (EC) as behavioral interventions for neonatal alcohol-related deficits (Hamilton et al., 2012, 2014, 2015; Schreiber et al., 2013). Both of these interventions are beneficial for measures of neuroplasticity and can alter BDNF levels. Thus, my second question for Aim 2 was: *does housing with access to wheel running or with access to wheel running followed by housing in a complex environment impact Bdnf exon-specific gene expression and DNA methylation in the adult hippocampus of neonatally alcohol-exposed or control rats?* Wheel running access for 42 days (WRWR) increased BDNF protein and gene expression in the PD72 hippocampus. Twelve days of wheel running followed by 30 days of housing in EC (WREC) did not affect BDNF. Additionally, WRWR was associated with less *Bdnf* exon I DNA methylation in control animals, though this effect was not seen in

AE rats. Thus, while both housing interventions enhance neuroplasticity, only WRWR results in increased BDNF levels and epigenetic alterations in the adult rat hippocampus.

Based on our lab's work demonstrating the negative impact of our model of FASD on the survival of adult-born dentate gyrus granule cells, further investigation of how neonatal alcohol exposure affects the various stages of adult neurogenesis was necessary. For Specific Aim 3, I asked *does neonatal alcohol exposure (PD4-9) alter the number of proliferating cells (Ki-67+), the number of immature neurons (DCX+), or the dendritic morphology of the DCX+ neurons in the adult rat hippocampus?* Alcohol exposure did not significantly impact the number of proliferating cells nor the number of immature neurons in the adult hippocampus. However, the dendrites of the immature granule cells were significantly less complex in AE animals compared to the control groups. Thus, it is likely that it will be more difficult for these neurons to make a sufficient number of excitatory synapses and functionally integrate into the hippocampal network, possibly leading to the decreased long-term granule cell survival observed in our model of alcohol exposure.

As our lab has successfully used WREC as a therapeutic intervention to rescue alcohol-induced decreases in dendritic complexity in the medial prefrontal cortex and enhance long-term cell survival in the dentate gyrus (Hamilton et al., 2012 2015), my second question in Aim 3 was: *does housing with access to wheel running or with access to wheel running followed by housing in a complex environment impact the number of proliferating cells (Ki-67+), the number of immature neurons (DCX+), or*

the dendritic morphology of the DCX+ neurons in the adult dentate gyrus of neonatally alcohol-exposed or control rats? In AE animals, both WREC and WRWR robustly enhanced dendritic complexity, thus reversing alcohol-induced deficits on this measure. In control animals, WRWR positively altered dendritic morphology while WREC had little effect. Changes to dendritic complexity were less robust in control animals compared to the AE group, suggesting a ceiling for neuroplastic benefit in the healthy hippocampus. Overall, these data support WREC and WRWR as behavioral therapies for alcohol-induced deficits in hippocampal neuroplasticity.

This dissertation sought to and successfully answered the questions posed above. However, for every answer found, more questions ultimately remained. Important future directions for this research line are still open. One of the most valuable questions remaining to be addressed in the role of sex in the current findings. This dissertation focused on male animals only and there is a plethora of evidence supporting further investigation of these measures in female rats. Literature using female animals has reported a variety of important findings relevant to the experiments discussed here, including differential effect of developmental alcohol exposure on LTP and BDNF in males and female rats (Sickmann et al., 2014; Titterness & Christie, 2010; Moore et al., 2004), different levels of cytokines and microglial number in the neonatal female and male brains (Schwarz et al., 2012), and distinct effects of early life stress on gene expression and DNA methylation in males and females (Blaze et al., 2013; Blaze & Roth, 2013; Roth et al., 2014). Based on these studies, it is likely that data from female animals would be quite different from

the results described here. This dissertation also supports the continued investigation of neuroplasticity across the lifespan. Additional time points between PD10 and PD72 would give valuable information given the transient nature of alcohol-related alterations to BDNF. Further, examination of other brain regions is critical, particularly the prefrontal cortex given our lab's previous work showing decreased dendritic complexity in this region (Hamilton et al., 2010, 2015). Finally, BDNF is just one neurotrophin in a symphony of plasticity-related proteins. Investigation of how our model of FASD impacts other proteins such as NGF, Arc, and EGR-1 is an important next step.

In conclusion, this dissertation demonstrated that third trimester-equivalent alcohol exposure negatively impacts dendritic complexity of immature neurons in the adult rat hippocampus, activates the neonatal neuroimmune response, and transiently alters levels of hippocampal BDNF. Further, these experiments support investigation of BDNF as an innate compensatory response to protect against the damaging effects of neonatal alcohol exposure and the use of exercise and housing in a complex environment as behavioral interventions to be implemented in adolescence or adulthood. Finally, these experiments help further our understanding not only of our rat model of FASD but of molecular and neuroanatomical changes possibly contributing to the cognitive and behavioral deficits afflicting children with FASD.

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Appendix

A INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORMS

University of Delaware
Institutional Animal Care and Use Committee
Request to Amend an Animal Use Protocol

RECEIVED
APR 30 2015
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Title of Protocol: Therapeutic Motor Training and Fetal Alcohol Effects

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

Principal Investigator: Dr. Anna Y. Klintsova

Requested Changes

I am requesting a change to: ***(Check all that apply)***

- ☐ Animal Species *(Complete Section 1)*
- ☒ Animal Numbers *(Complete Section 2)* X
- ☐ Animal Procedures *(Complete Section 3)*
- ☐ Therapeutic or Experimental Agents *(Complete Section 4)*
- ☐ Pain Category *(Complete Section 5)*
- ☐ Use of Biological Material, Hazardous Agents or Radiation *(Complete Sections 4 & 6)*
- ☐ Other *(Specify)* Click here to enter text.

(Complete Section 7)

Changes MUST NOT be initiated until IACUC approval is granted

Official Use Only

IACUC Approval Signature: Glen Tellam, DVM

Date of Approval: 5/14/15

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 Klimkova _____ 04-24-2015____ 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	
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