INFLUENCE OF WHEEL RUNNING AND ENVIRONMENTAL COMPLEXITY ON ADULT HIPPOCAMPAL NEUROGENESIS IN RATS POSTNATALLY EXPOSED TO ALCOHOL

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

In humans, exposure to alcohol during prenatal development can lead to Fetal Alcohol Spectrum Disorders, which cause a multitude of detriments to the central nervous system that persist throughout the life of an individual. One brain region particularly susceptible to alcohol exposure during the third trimester of pregnancy is the hippocampus, an area critical for learning, spatial memory and overall cognition. A potential mechanism by which developmental alcohol exposure may interfere with hippocampal structure and function is through the alteration of the propensity for adult neurogenesis, which is the continuous generation of neurons in the adult brain. Previous work in our lab has demonstrated that alcohol exposure impairs new cell survival in the adult hippocampus, and then, differentially, that behavioral interventions (voluntary exercise and environmental complexity) attenuate alcohol-induced deficits to adult neurogenesis. The current study seeks to investigate how immature neurons within the dentate gyrus respond to an insult of alcohol incurred during development, and how the beneficial interventions of wheel running and environmental complexity affect the brain into adulthood. For these experiments, animals were exposed to alcohol on postnatal days (PD) 4-9 (5.25 g/kg/day) and then housed in one of three different conditions from PD30-72: social housing (SH), voluntary wheel running (WR/WR), and wheel running (12 days) followed by

environmental complexity (30 days; WR/EC). In SH animals, alcohol exposure did not affect baseline number of doublecortin-positive (DCX+) cells, which was consistent with our expectations, while WR/WR, but not WR/EC, successfully increased the number of DCX+ cells in suckle control (SC) and sham-intubated (SI) animals compared to SH animals. In addition, no baseline alterations to total amount of dendritic material were found in the AE group. WR/WR, but not WR/EC, increased the total dendritic material within SC and SI animals. Interestingly, an increase in dendritic material was not observed within AE animals, suggesting that AE animals are not gaining as much benefit from WR/WR on this measure as the control animals. These results confirm that aerobic exercise is a positive intervention as measured by adult hippocampal neurogenesis. Additionally, it is possible that total dendritic material is negatively affected enough by alcohol exposure in a way that disrupts the ability of these cells to be affected exercise as profoundly.

INTRODUCTION

1.1 Fetal Alcohol Spectrum Disorders

Alcohol has been established as an exceedingly detrimental teratogen, and exposure to alcohol during periods of fetal development causes a myriad of detriments to the central nervous system (CNS) that persist throughout the lifetime of an individual. Exposure to alcohol *in utero* can lead to the development of Fetal Alcohol Spectrum Disorders (FASD), an umbrella term which encompasses a variety of alcohol-related disorders. The most severe of these disorders is Fetal Alcohol Syndrome (FAS), which is classified by stereotyped neurobehavioral deficits, facial dysmorphologies such as a smooth philtrum and a flat face, growth retardation, and damage to the CNS, resulting in low brain weight and volume, and simplified cortical structure, as manifested by flattening of the cortical surface, leaving less prominent sulci and gyri (Riley, Infante, & Warren, 2011). In addition, those with FASDs place a large stress upon the economy; it is estimated that the lifetime cost for one individual with FAS is $2 million, with an annual cost of over $4 billion cumulatively in the United States (Lupton et al., 2004).

Deficits in neurological and behavioral functioning such as lower overall IQ; impairments in learning and memory, language, attention, and executive functioning; and disrupted social and adaptive functioning resulting from FASD persist in affected individuals throughout life (Mattson & Riley 1998). These deficits can lead to the development of secondary disabilities and make it difficult for an individual to

maintain societal independence (Clarke & Gibbard 2003). Because FAS and FASD affect such a broad spectrum of human neurological function and cause long-lasting and severe effects, the pathology resulting from developmental alcohol exposure must be more thoroughly understood.

1.2 Adult Neurogenesis and hippocampal-dependent learning and memory

A potential mechanism by which developmental alcohol exposure may interfere with hippocampal structure and function into adulthood is through the alteration of the process of adult neurogenesis, which is the continuous generation of neurons in the adult brain. Adult neurogenesis has been consistently found in two brain regions in many species: the subventricular zone of lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus (Altman, 1965; Emsley et al., 2005; Eriksson et al., 1998; Kheirbek and Hen, 2013). Within the DG, adult neurogenesis begins with the proliferation of stem cells within the SGZ that divide into neural progenitor cells (NPCs). During the first week after initial proliferation, NPCs begin migration outward into the granule cell layer of the DG and continue to mature into immature neurons. As immature neurons, the cells grow neuritis out towards the molecular layer and entorhinal cortex. However, the immature neurons do not integrate into the hippocampal network until about 3 weeks after initial proliferation, at which point they form afferent connections to CA3 and efferent connections to the entorhinal cortex. Following integration, the neurons continue to

develop until 4-6 weeks of age, at which point they are fully integrated into the surrounding hippocampal network and display fully mature dendritic trees (reviewed in Deng et al., 2010).

The stages of adult neurogenesis can be determined using antibody markers for specific proteins expressed during the varying stages of cell maturation, including markers to track progression through the cell cycle, neuroblasts, immature neurons, and fully mature granule cells. Doublecortin (DCX) is a microtubule-associated protein expressed in immature neurons and is often used as a marker of adult neurogenesis. DCX is expressed soon following initial cell division once cells commit to a neuronal fate and continues to be expressed throughout maturation (2-3 weeks). DCX expression ceases once the immature neurons have developed mature processes and begun to integrate into the hippocampal network.

Adult neurogenesis in the DG occurs for the duration of the lifespan, and newly generated neurons have been shown to contribute to hippocampal-dependent learning and memory (Shors et al., 2001), including tasks involved with pattern separation and spatial learning, such as the Morris Water Maze. It has been proposed that newly generated granule cells in the DG may function as modulators of principal neurons to influence pattern separation (Sahay et al., 2011), a process by which overlapping or similar inputs are transformed into less similar outputs (Colgin et al., 2008).

Previous literature supports the idea that hippocampal-dependent learning selectively aids survival of newly born neurons within the DG. Learning of tasks that

require the hippocampus (such as the Morris Water Maze) increases the number of adult-born granule cells in the DG when cells have matured for about 1 week, but this effect is not seen for non-hippocampal-dependent tasks (reviewed by Deng et al., 2010). Acquisition of spatial memory tasks, such as the Morris Water Maze, promoted survival of granule cells that underwent proliferation 7 days prior to water maze training (Dupret et al., 2007). Additionally, work from our lab has shown that alcohol exposure during the early neonatal time period impaired trace eyeblink conditioning (hippocampal and cerebellum-associated task) relative to control animals and deficits in survival of adult-born neurons (Hamilton, Jablonski, et al., 2014). While the impairment in adult neurogenesis was not directly tied to deficits in trace eyeblink conditioning performance in this study, successful acquisition of trace eyeblink conditioning relies on a functional hippocampus, suggesting that disruptions to adult neurogenesis could impair learning on this task.

1.3 Effects of developmental alcohol exposure on hippocampal neuroanatomy and Adult Neurogenesis

The severity and manifestation of teratogenic effects caused by developmental alcohol exposure varies greatly depending on the timing, pattern, and dose of exposure, both in humans and in rodent models (Bonthius & West, 1990; Cartwright & Smith, 1995; Coles, 1994; Maier & West, 2001; Miller, 1992). One specific period of development during which alcohol exposure causes particularly robust cognitive and behavioral deficits in humans and rodents is the “brain growth spurt”, a period of

mass neuronal differentiation, maturation, and synaptogenesis in many neural structures, such as the hippocampus, cerebellum, and several cortical areas. The brain growth spurt corresponds with the end of the third trimester in humans and the first two postnatal weeks in rats (Dobbing & Sands, 1979); alcohol exposure during this period has been shown to grossly affect these brain structures in rodent models (Pauli, Wilce, & Bedi, 1995; Pierce, Goodlett, & West, 1989; Klintsova et al., 2007). Alcohol exposure during the brain growth spurt is known to be detrimental towards adult brain plasticity (reviewed in Hannigan et al., 2007), including measures of learning, memory, and hippocampal LTP.

Several studies have examined the effect of neonatal alcohol exposure adult hippocampal neurogenesis. Whether enduring changes in adult neurogenesis can result from alcohol exposure during the brain growth spurt has not been fully explored, and reported effects of alcohol exposure in rat models vary from no changes (Choi et al., 2005) to significant decreases in adult neurogenesis (Redila et al., 2006). A single injection of 20% ethanol (5 g/kg) on postnatal day (PD) 7, decreased proliferation of new cells and the number of neuronal progenitors in the dorsal DG when evaluated during adulthood (Ieraci and Herrera, 2007). Furthermore, previous work in our lab has demonstrated that binge-like alcohol exposure during the third trimester-equivalent did not alter cell proliferation on PD50 but decreased survival of these newly generated neurons on PD50 and PD80 (Klintsova, et al. 2007). Additionally, a third study showed that baseline cell proliferation was not affected by PD4-9 alcohol exposure and that voluntary exercise (wheel running [WR]) during adolescence can

induce comparable increases in cell proliferation in alcohol-exposed and control rats; however, long-term survival of the newly generated cells was compromised in alcohol-exposed rats (Helfer et al., 2009). In contrast to the results of the study conducted by Helfer and colleagues, PD1-10 alcohol exposure altered cell proliferation in rats during adolescence and increased early neuronal maturation but had no effect on cell survival (Boehme et al., 2011), suggesting that timing of the alcohol exposure and age when the tissue is harvested (adolescence vs. adulthood) could impact results regarding how neonatal alcohol exposure impact adult neurogenesis. Overall, these studies indicate that neonatal alcohol exposure appears to negatively influence certain stages of adult neurogenesis in the DG, though the exact mechanisms underlying these impairments are not known. It is important to examine further the impact of developmental alcohol exposure on the specific cell populations of the DG, as well as investigate potential behavioral therapies by which to rescue deficits in adult neurogenesis, as it is critical for proper hippocampal function and an important mechanism in processes of learning and memory.

1.4 Effects of voluntary exercise and environmental complexity on Adult Hippocampal Neurogenesis

While exposure to teratogens can produce deficits in adult neurogenesis, other environmental factors can upregulate proliferation of new cells in the DG and enhance cell survival. Voluntary exercise and housing in a complex environment have both been shown to enhance measures of hippocampal neuroplasticity, including adult

neurogenesis. Rates of adult neurogenesis in the adult mouse were first shown to be affected by voluntary exercise by van Praag and colleagues (1999). Since that discovery, much work has been directed towards understanding the positive effects that exercise has upon adult neurogenesis.

Boehme and colleagues (2011) found that exercise enhanced cell proliferation, neuronal maturation, and cell survival in control, pair-fed, and alcohol-exposed rats. As mentioned previously, previous work from our lab has also shown that 12 days of voluntary exercise (wheel running) increases cell proliferation in alcohol-exposed and control rats, but the long-term survival of these newly generated cells was compromised in alcohol-exposed rats (Helfer et al., 2009). However, following the wheel running by 30 days of exposure to a complex environment (environmental complexity; EC) successfully promoted the survival of adult-generated neurons in alcohol-exposed rats back to control levels (Hamilton et al., 2012).

Environmental complexity was originally defined as a combination of complex inanimate and social stimulations (Rosenzweig et al., 1978) and is known for its powerful effects upon brain plasticity. Previous studies have shown that exposure to EC can increase brain weight and size, number of dendritic bifurcations, dendritic length, spine density, and cell survival in control animals (Darmopil et al., 2009; Gelfo et al., 2009; van Praag et al., 2000). This information taken together suggests that a treatment of exercise followed by EC may significantly improve alcohol-related deficits in hippocampal adult neurogenesis.

The present study aims to investigate whether binge-like third trimester-equivalent alcohol exposure affects early stages of granule cell maturation in the subgranular zone and the granule cell layer of the dentate gyrus of the adult rat hippocampus. Additionally, this study assesses whether the interventions of exercise (wheel running), or exercise followed by housing in environmental complexity alters the number of DCX+ neuronal progenitor cells and immature neurons in the adult dentate gyrus. I will also assess how these two factors (neonatal treatment and adolescent housing condition) affect total dendritic material as an indirect measure of dendritic complexity.

Based on the current literature and previous work from our lab, we expect that that binge-like exposure to alcohol during the third trimester-equivalent will not affect number of DCX+ cells across dosing conditions, but will decrease dendritic complexity. Additionally, we expect that voluntary exercise and voluntary exercise followed by exposure to a complex environment will increase the number of DCX+ cells across all dosing conditions, and also rescue deficits in dendritic complexity seen within alcohol-exposed animals. It is possible that neonatal alcohol exposure or access to the different housing conditions could affect neuronal migration of the immature neurons from the SGZ into the GCL. To assess changes to neuronal migration, the percentage of cells within the SGZ as compared to the GCL was analyzed.

Chapter 1

METHODS

2.1 Animal Subjects

Timed-pregnant Long Evan's dams were ordered from Harlan and housed individually in standard cages until they gave birth. On postnatal day 3 (PD3), litters were culled to 8 pups, with six males and two females in each litter, and marked sub-dermally with non-toxic black ink for purposes of identification. On PD4, rat pups were pseudo-randomly assigned to one of three dosing conditions: alcohol-exposed (AE/ n=12), sham-intubated (SI/ n=13), or suckle control (SC/ n=16). SC animals were generated from a separate litter. Only male animals were used in the experiments described below. A visual depiction of the experimental timeline is shown below in Figure 1. All procedures were carried out in accordance with the animal use protocol approved by University of Delaware Institutional Animal Care and Use Committee.

2.2 Alcohol Exposure

On PD days 4-9, AE animals received a dose of ethanol through intragastric intubation in a binge-like manner (5.25 g/kg/day; 2 doses, 2 hrs apart), and SI animals were intubated without milk or ethanol to control for the stress of the intubation

procedure. The intragastric intubation procedure involves passing a small, flexible plastic tube lubricated with corn oil down the esophagus into the stomach of the pup. Alcohol was administered as 11.9% (v/v) solution in artificial milk formula. SC pups remained undisturbed with the exception of daily weighing to ensure proper development. Blood samples were collected from the tail vein, by manner of a tail clip, of AE and SI pup 90 min after the second alcohol dose on PD4. Samples were centrifuged for 15 minutes at 15000 revolutions/min, the plasma was collected, and the samples were stored at -20°C. Blood alcohol concentration was assayed from the plasma of each animal's blood sample using an Analox GL5 Alcohol Analyzer (Analox Instruments, Boston, MA).

2.3 Housing Conditions

On PD23, rat pups were weaned from their mothers, and left undisturbed in standard housing (2-3/ cage) until PD30. From PD 30-72, rats were randomly assigned to one of three housing conditions: standard social housing (SH; 3/cage), continuous wheel running (WR/WR; 3/cage), or 12 days of WR followed by 30 days of EC (WR/EC; 9-12/EC cage) (Experimental Timeline; Figure 1). During the WR intervention, animals lived in standard cages with free 24-hr access to stainless steel running wheels. Number of wheel revolutions was recorded via a mechanical clicker on each wheel and distance ran was recorded every day at 9:00AM. The EC intervention consisted of a 30″ x 18″ x 36″ galvanized 3-story steel cage (model: R-

695; cagestore.com) with 3 ramps, 2 balconies, a full middle floor, and a drop-in 3½-inch plastic pan filled with bedding. Cages were diversified with various objects, including standard pet toys, buckets, and blocks, and were switched out every other day.

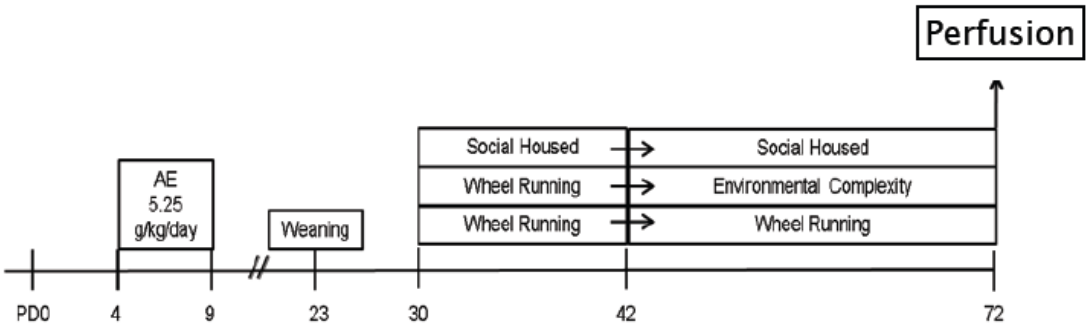


Figure 1 Experimental Timeline

2.4 Tissue Preparation

On PD72, animals were deeply anesthetized and transcardially perfused with heparinized 0.1 M phosphate-buffered saline (PBS; pH 7.2) followed by 4% paraformaldehyde in PBS (pH 7.2). Brains were stored at 4°C in 4% paraformaldehyde made in PBS for 24 hrs, transferred to 30% sucrose in 4% paraformaldehyde in PBS for 48 hrs, and then transferred to a new solution of 30% sucrose in 4% paraformaldehyde in PBS. Brains were sectioned horizontally at 40 micrometers on a cryostat and serial sections were collected maintaining order throughout the hippocampus so the entire extent of the hippocampus was collected.

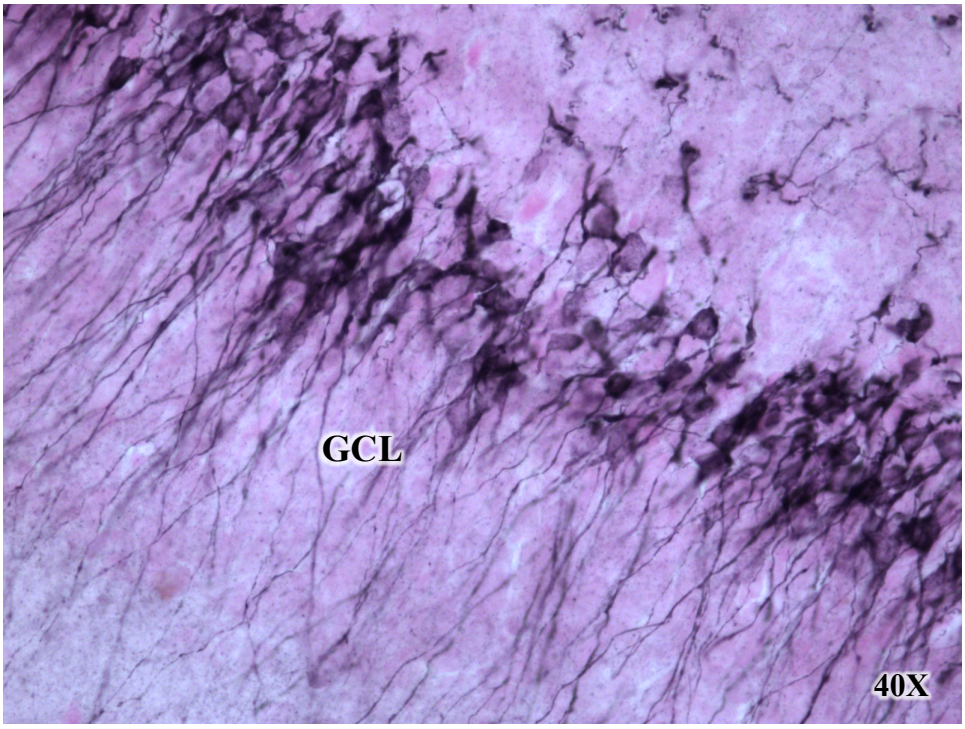
Sections were stored at -20°C in cryoprotectant solution (30% sucrose and ethylene glycol in 0.1M PBS).

2.5 Doublecortin Immunohistochemistry

Starting at around the beginning of the dentate gyrus, every 16th section (6-8 sections for every animal) was collected for purposes of immunohistochemical staining; sections were selected from the entire extent of the entire dentate gyrus. To analyze the number of cells positively stained for doublecortin (DCX), sections were rinsed 3 x 5 min in 0.1M Tris-buffered saline (TBS), then treated with 0.6% H2O2 in TBS to exhaust endogenous peroxidase activity for 30 min. Sections were washed again in TBS 3 x 5 min, then incubated in primary antibody made in blocking solution (3% normal donkey serum, 0.1% Triton-X in TBS) for 24 hrs at 4°C [1:500, goat anti-DCX C-18, Santa Cruz Labs]. Following incubation, tissue was rinsed 3 x 5 min with TBS then incubated in secondary antibody [1:250; biotinylated anti-goat (Vector Labs; Burlingame, CA, U.S.A)] for 1 hr at room temperature. After this, tissue was rinsed in TBS 3 x 5 min, incubated in avidin-biotin complex (ABC) diluted in blocking solution (Vector Laboratories, Burlingame, CA) for 1 hr, and rinsed again in TBS 3 x 5 min. Detection of the reaction was carried out using nickel-enhanced DAB as a chromegen (Hamilton et al., 2011). Stained tissue was then mounted on slides, left to dry for 48 hrs, counterstained with Pyronin-Y, then cover-slipped with DPX mounting solution

(Sigma-Aldrich; St. Louis, Missouri). Representative images of Doublecortin are below in Figure 2.

A



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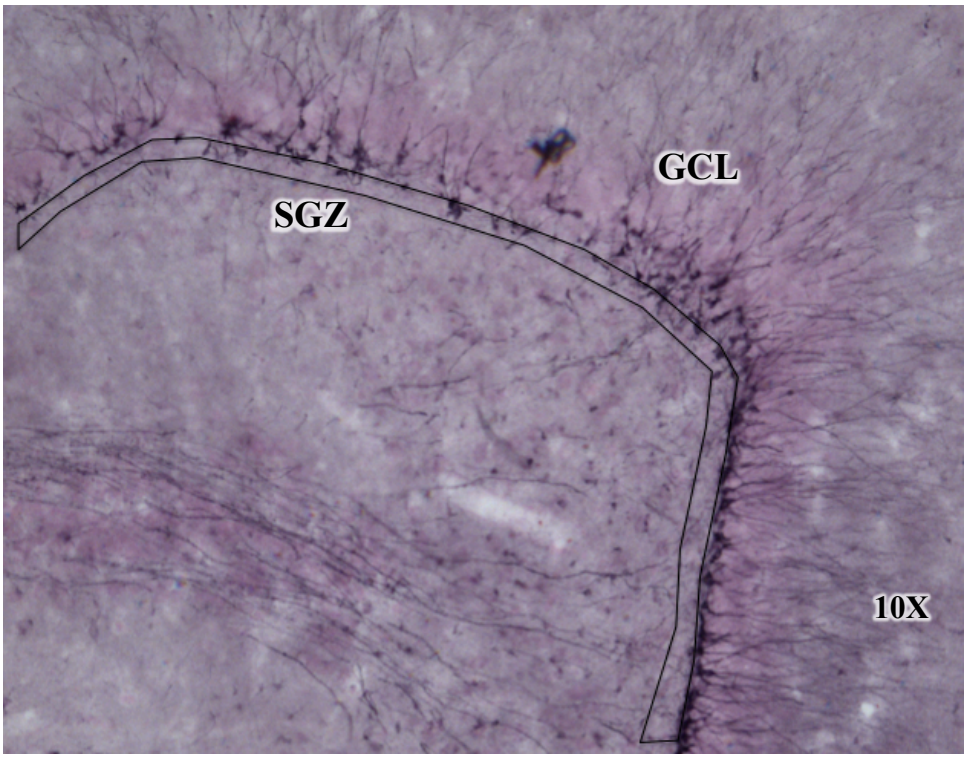

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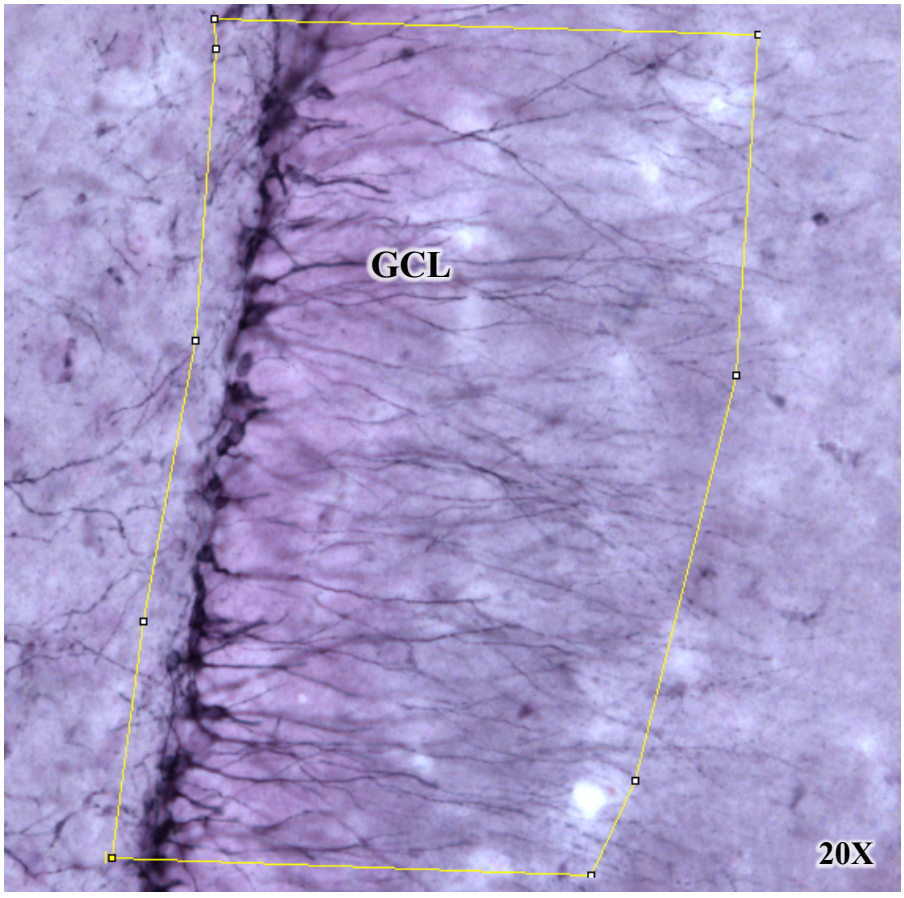

Figure 2 Representative images of dentate gyrus immunohistochemically stained for DCX from a SC-WR/WR animal. DCX+ cells are darkly stained and found along the border of the granule cell layer (GCL) and subgranular zone (SGZ). The GCL, which is indicated in (A) and (B), is the area of tightly-packed granule cells stained pink with Pyronin-Y. The SGZ is defined as the area immediately inside of the GCL and spans the width of to two cell bodies' diameter in from the GCL. The outline of the SGZ is indicated by the black line in (B). (A) was taken at a magnification of 400x (40x lens), and (B) was taken at a magnification of 100x (10x lens). (C) shows a representative image that would be used in optical densitometry analysis. The yellow line represents the area that would be traced for analysis, which is defined as the SGZ and the GCL plus the approximate distance of the width of the GCL traced in the direction of the molecular layer. (C) was taken at 200X magnification (20x lens).

2.7 Unbiased Stereology

Estimate of the total number of the DCX+ stained cells were made in an unbiased manner within a known volume of the dentate gyrus using the optical fractionator probe (Stereo Investigator, MBF Bioscience,Williston, VT). The StereoInvestigator software calculates the total volume of the outlined brain region taking into consideration the number of sections (section sampling fraction, ssf = 1/16) within the structure of interest, the thickness of the sections, and the number of the sampling sites within the dentate gyrus on each section. The grid frame was set to 200 X 200 micrometers and the counting frame set to 100 X 100 micrometers. A guard zone of 2 micrometers and a dissector height of 12 micrometers were used. Section thickness was measured at every 5^{th} counting site. An average section thickness was computed by the software and used to estimate the total volume of the DG sample region and the total number of DCX+ cells (thickness sampling fraction, tsf = # micrometers/ section thickness). The mean coefficient of error (CE) for the number of cells did not exceed the recommended 0.1. The total number of cells was estimated using the following formula:

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

2.8 Image Acquisition

To assess total dendritic material of the DCX+ cells, images of the dentate gyrus were analyzed using ImageJ software. Horizontal sections stained for DCX were examined and photographed using a light microscope. Two or three pictures were taken of each section that contained the dorsal dentate gyrus with a 20x lens (200x magnification). Horizontal sections were qualified as containing either dorsal or ventral hippocampus based on the presence of the medial portion of the DG. Moving ventrally down the dorsoventral axis, sections were considered dorsal if the medial DG persisted, and ventral if it was absent. This transition occurred at approximately -4.5 mm Bregma (Paxinos & Watson, 1998). Pictures were generally taken of the same area within each section to reduce the chance that areas of the dentate gyrus were picked that had a higher or lower density of DCX+ cells. All images were captured at the same brightness level and camera settings using StereoInvestigator.

2.9 Optical Densitometry

Upon acquiring the images, ImageJ software was used to determine the number of DCX+ cells per unit area (μm^2) of examined dentate gyrus through the ratio

of pixel luminescence of individual stained cells to that of the background. The dentate gyrus and molecular layer were traced in the image to restrict the total area considered to that of the dentate gyrus, subgranular zone, and molecular layer. The subgranular zone was traced to include two cell widths to the inside of the granule cell layer, and the molecular layer was represented by tracing outwards from the granule cell layer to a width twice that of the granule cell layer. Images were converted to 16-bit grayscale, 50 units of brightness were subtracted, and the sharpen tool of the software was used. These steps were included in the procedure in order to enhance the signal to noise ratio of DCX+ cells/DCX- cells. Following these adjustments, the experimenter, who is blind to the experimental condition of each animal, then sets a threshold for pixel luminescence at which all DCX+ cells and corresponding dendrites appear, but no DCX- cells are counted. The threshold set by the experimenter is then applied by the program, which produces a new image that contains only cells with pixel luminescence below the threshold. The experimenter traces the area of the section to be analyzed, and then ImageJ measures the Mean Gray Value of the selected area, which is the amount of dark pixels within a selected area divided by the total number of pixels. The Mean Gray Value is the value used to compare optical density between groups of animals. Data is represented as percent of control of staining batch to account for the differences seen in staining intensity between staining batches of DCX.

2.10 Statistical Analysis

For animal weights, a repeated-measured analysis of variance (ANOVA) with the factors of Day and Neonatal Condition was used. For PD4-9 weights, a one-way ANOVA with the independent variable of neonatal condition was used. For PD30-72, a one-way ANOVA with the independent variable of housing was used. For cell counts and dendritic material, two-way ANOVAs with the factors of housing condition and neonatal treatment were performed to analyze for main effects. Planned one-way ANOVAs were performed to analyze for differences within dosing groups. Tukey's *post-hoc* tests were performed to confirm statistical differences within dosing groups. Outliers were removed using Grubb's test. Statistical significance was considered to be $p < 0.05$; trending differences of $p < 0.1$ are also discussed when appropriate.

Chapter 2

RESULTS

3.1 Weights, blood alcohol concentrations, and wheel running

Animals weights for PD4, PD10, PD30, PD42, and PD72 are displayed in Table 1, which shows the average weights for each condition at each date the animals were weighed, and are pictured graphically in Figure 1. All animals continued to gain weight throughout treatments (Table 1) and placement within various housing conditions (Table 2). The influence of postnatal treatment on body weights was determined using a repeated-measures ANOVA with post-natal treatment (AE, SI, SC) as the between subject factor and post-natal day as a within-subject factor. There was an observed main effect of day upon animal weight; all animals gained weight from PD4-9 ($F_{(1,61)}$=2865.683, $p < 0.001$). Across PD4-9, there was a main effect of neonatal treatment ($F_{(2,61)}$=36.833, $p < 0.001$); AE animals weighed less than SH and SI animals on PD9 (Figure 3). The influence of housing condition upon body weight was determined using a repeated-measures ANOVA. Weight differences between neonatal treatment had disappeared by PD30. There was an observed main effect of day upon animal weight; all animals gained weight from PD30-72 ($F_{(2,51)}$=5217.082, $p < 0.001$). Across PD30-72, there was a main effect of housing condition upon weight,

WR/WR animals weighed less than SH and WR/EC animals on PD72 ($F_{(2,61)}$=2865.683, $p < 0.001$). Average BACs were obtained using blood samples taken after the first day of AE (PD4) (Table 1).

The average distance run by all animals from PD31-42 was 2.86 miles ± 1.18 (standard deviation; SD) per 24 hrs. The distance run by all animals within the WR/WR condition from PD43-72 was 9.2 miles ± 3.71 per 24 hrs (Figure 4).

Table 1 Animal weights (grams ±SD) of SC, SI, and AE dosing conditions on PD4 and PD9and BACs (mg/dl ±SEM) of AE pups on PD4. SC: Suckle Control; SI: Sham-intubated; AE: Alcohol-Exposed. *indicates significant difference ($p < 0.05$) from SC; group.

Dosing condition	Body Weight (g ± SD)		BAC (mg/dl ±SEM)
	PD4	PD9	
SC	10.6 (±.2)	20.2 (±.3)	n/a
SI	10.7 (±.2)	19.8 (±.3)	n/a
AE (5.25 g/kg/day)	10.7 (±.2)	17.15 (±.4)*	334.3933 ± 25.9

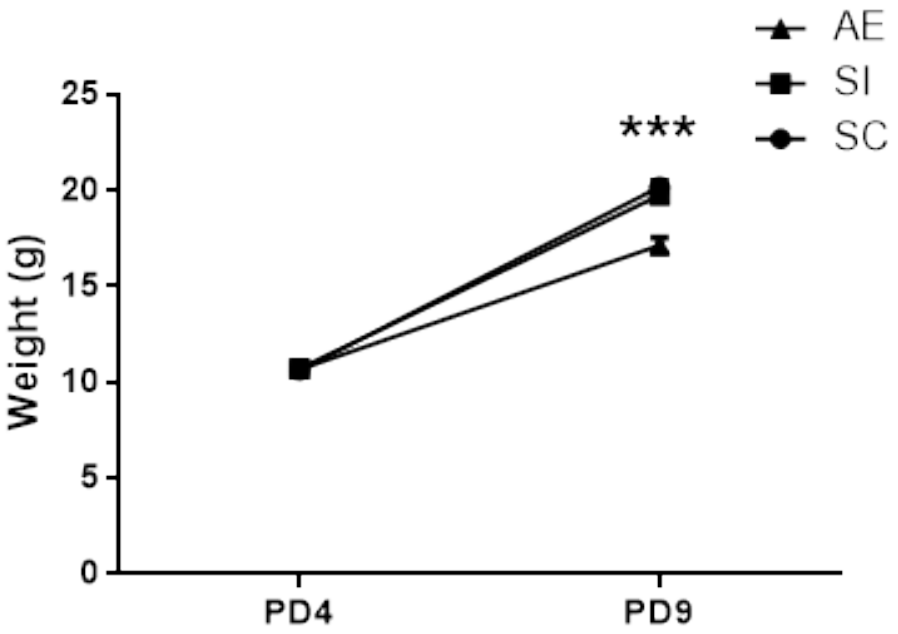

Figure 3 Animal weights of SC, SI, and AE dosing conditions on PD4 and PD9. AE animals weighed significantly less than SC and SI animals on PD9.

Table 2 Animal weights (g, (±SE)) of SH, WR/EC, and WR/WR housing conditions on PD30, PD42, and PD72. On PD72, WR/WR animals weighed significantly less than SH and WR/EC animals. ($p < 0.001$)

Housing Condition	Body Weight (g ± SD)		
	PD30	PD42	PD72
SH	100.6 (±1.3)	198.4 (±3.0)	387.2 (±5.0)
WR/EC	98.9 (±1.9)	196.4 (±4.6)	382.7 (±4.6)
WR/WR	100.4 (±1.2)	191.9 (±3.0)	364.6 (±5.3)

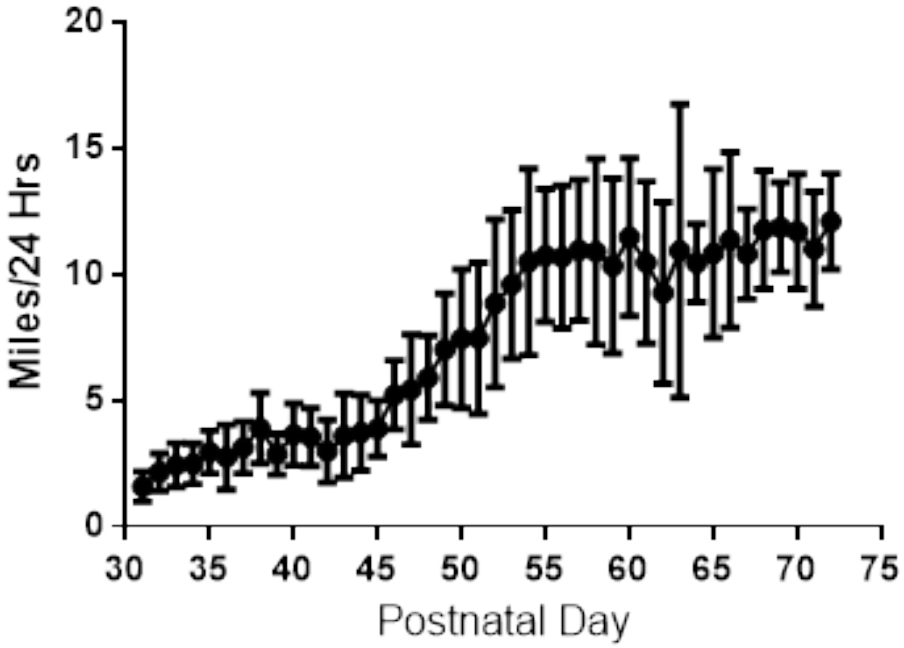

Figure 4 WR averages across the days (mean miles ± SD). PD31-42: 2.86 ± 1.18; PD43-72: 9.2 ± 3.71.

3.2 DCX+ Cell Counts

A two-way ANOVA examining effects of housing condition and neonatal treatment upon cell number was run and revealed a main effect of housing on DCX+ cell counts in the DG ($F_{(2,34)}$=9.844, $p < 0.001$). Then, planned one-way ANOVAs analyzing total DCX+ cell number across dosing conditions revealed main effects within SC ($F_{(2,15)}$=5.384, $p = 0.020$) and a trending main effect within SI ($F_{(2,14)}$=3.690, $p = 0.056$) dosing conditions, but none within AE ($F_{(2,11)}$=2.008, $p = 0.190$). Within the dentate gyrus, a significant increase of cell number was seen in

WR/WR group as compared to SH and WR/EC. Within the SC neonatal treatment condition, a Tukey post-hoc test confirmed a significant difference between WR/WR and SH groups (*p=0.019)*, and a trending difference between WR/EC and SH groups ($p = 0.063$) (Figure 5A). Within the SI condition, a Tukey's post-hoc test showed a trending difference between SH and WR/WR groups *(*$p = 0.053$). There were no main effects of housing seen within the AE group. These results indicate that there may be an effect of voluntary exercise upon adult neurogenesis in the dentate gyrus (Figure 5A). A two-way ANOVA revealed no significant main effect of housing ($F_{(2,34)}=1.751$, $p = 0.189$) or neonatal condition ($F_{(2,34)}=0.192$, $p = 0.826$) upon percentage of cells in the SGZ as compared to the GCL (Figure 5B).

These results were consistent with expectations, in that they indicate that exposure to alcohol did not decrease the number of DCX+ cells, but wheel running did successfully increase the number of neuronal progenitors and immature neurons within the DG in control animals. Additionally, neuronal migration was not affected by neonatal treatment or housing condition. The combined intervention of WR/EC did not have any affect upon DCX+ cell number.

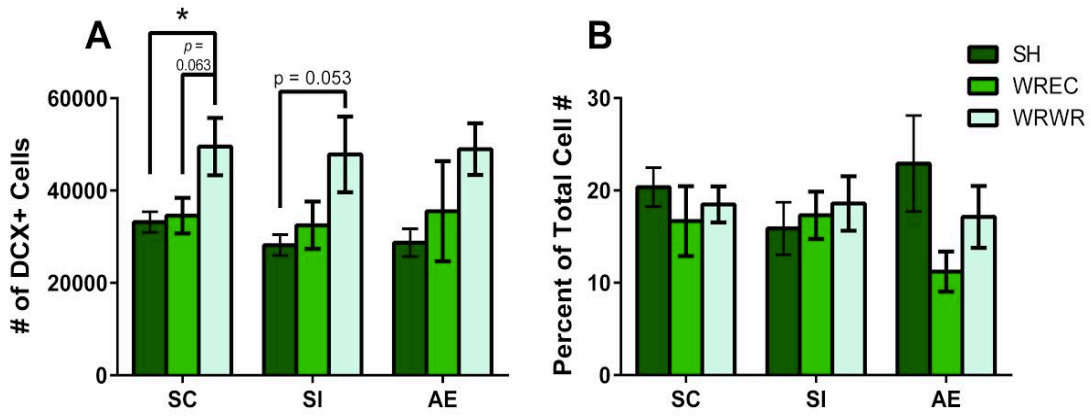

Figure 5 Cell counts of DCX+ stained cells in dentate gyrus. (A) Within the SC group, the WR/WR intervention significantly increased cells stained positively for DCX as compared to SH (p=0.019), with a trending increase present between WR/WR and WR/EC (p=0.063). Within the SI group, the WR/WR intervention encouraged a trending increase in DCX+ cell number between WR/WR and SH groups (p=0.53). No significant differences existed within the AE group.

3.3 Dendritic Material in Dorsal Dentate Gyrus

A two-way ANOVA examining DCX+ cell density (DCX+ cells/μm^2) found no significant main effect across dosing conditions ($F_{(2,29)}$=2.274, p = 0.102); however, a main effect of housing was found ($F_{(2,29)}$=9.311, p = 0.001). One-way ANOVAs analyzing DCX+ cell density within dosing conditions revealed main effects within SC ($F_{(2,15)}$=7.766, p = 0.006) and SI ($F_{(2,12)}$=8.694, p = 0.006) groups, but not within the AE dosing condition ($F_{(2,8)}$=0.047, p = 0.954). Then, Tukey's post-hoc analysis confirmed that there were statistically significant differences between the

WR/WR group as compared to WR/EC ($p = 0.011$) and SH ($p = 0.012$) groups for the SC dosing condition. These differences were mirrored within the SI condition for both WR/WR ($p = 0.013$) and WR/EC ($p = 0.020$) as compared to SH. However, there were no statistical differences seen for the AE dosing condition (Figure 6).

As expected, WR did increase dendritic complexity in SH and SI groups. It was expected that alcohol exposure would decrease dendritic complexity as compared to SC and SI groups, but this was not observed. Instead, the results show that WR, though it has been shown in other studies to increase dendritic complexity in AE animals back to normal levels, was not able to increase dendritic complexity within AE animals in the present study.

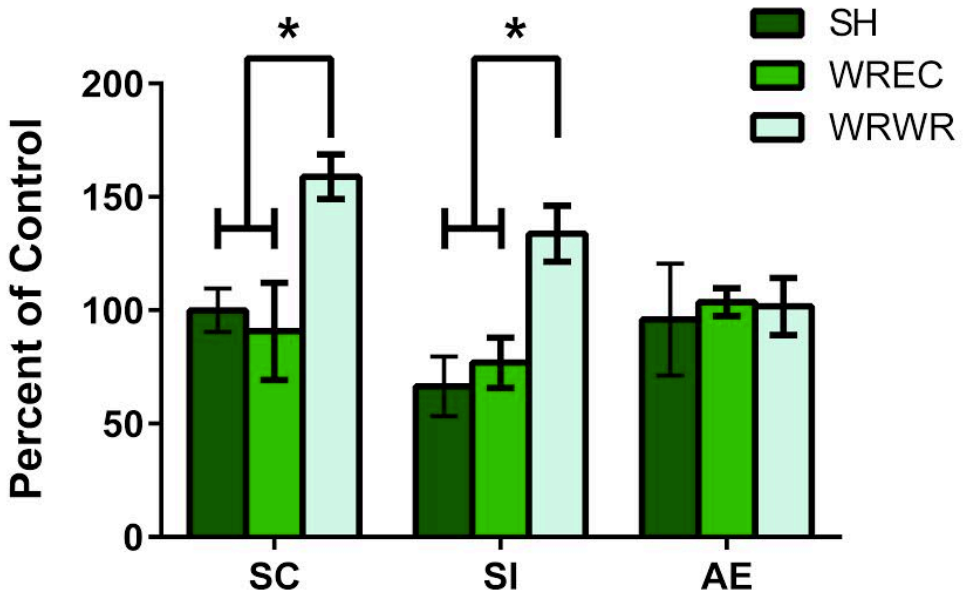

Figure 6 Optical Density of DCX+ stained cells in the dorsal dentate gyrus. Within the SC group, the WR/WR intervention increased the optical density of DCX+ cells as compared to SH (p=0.012) and WR/EC (p=0.011) groups. These differences were similarly seen within the SI group; WR/WR increased the optical density of DCX+ cells as compared to SH (p=0.013) and WR/EC (p=0.020) groups. No significant differences existed for the AE dosing condition.

Chapter 3

DISCUSSION

4.1 Summary of Results

The current study analyzed the effects of neonatal alcohol exposure and environmental interventions during adolescence/ early adulthood upon immature neuron cell number and dendritic complexity within the dentate gyrus of the hippocampus of the adult rat brain. First, as expected, we discovered that neonatal alcohol exposure had no effect upon the number of doublecortin (DCX) –positive neuronal progenitors and immature neurons seen within the dentate gyrus. Additionally, migration of cells from the SGZ into the GCL was not affected by alcohol exposure or housing as no differences in the ratio of cells within the SGZ/GCL were shown. However, we did find that an intervention of wheel running (WR/WR), but not wheel running followed by environmental complexity (WR/EC), increased the number of DCX+ cells for suckle control (SC) and sham-intubated (SI) conditions, but not in the alcohol-exposed (AE) animals. Furthermore, this study found that WR/WR, but not WR/EC, increased total amount of dendritic material in DCX+ cells in the SC and SI groups, but not in the AE group. It was expected that AE would cause a decrease in total dendritic material that would then be rescued back to control levels

by WR/WR or WR/EC, as has been previously demonstrated by our lab in pyramidal cells in the medial prefrontal cortex (Hamilton et al., 2010). Contrary to this expectation, neonatal alcohol exposure had no effect upon baseline total dendritic material in immature neurons. Additionally, it was expected that the WR/WR housing condition would increase the number of DCX+ cells regardless of neonatal condition, as has been shown previously (Boehme et al., 2011). The results found in this study suggest that interventions previously shown to rescue alcohol-induced deficits seen in adult neurogenesis in other models of FASD may not be leading to an increase in the number of immature neurons and the complexity of those neurons at this specific time point of postnatal life (PD72) or at this specific time point of adult neurogenesis (DCX+ immature neurons) when using our particular model of developmental alcohol exposure.

4.2 DCX Cell Count

Increases in number of DCX+ cells were seen in the DG following the WR/WR intervention. Previously, wheel running has been shown to be an immensely beneficial environmental intervention in regards to measures of neuroplasticity such as neurogenesis and dendritic complexity (Boehme et al., 2011; Hamilton et al., 2012; van Praag et al., 2009). Studies conducted by our own lab have found that 12 days of voluntary exercise significantly increased survival of adult-born neurons (Helfer et al., 2009). This enhancement in new cell survival occurs even when rats have been

postnatally exposed to alcohol during the third trimester-equivalent. Exercise-induced increases in adult neurogenesis within alcohol-exposed rats has also been demonstrated in the work of Boehme and colleagues (2011), who found that voluntary exercise enhanced proliferation, maturation, and cell survival in rats that were exposed to alcohol during the third trimester-equivalent. It is likely that we would see an increase in the number of DCX+ cells within the AE-WR/WR group compared to the AE-SH group if more animals could have been added to this group (current n=7), as an increase in cell number can be seen visually when the data is represented graphically (Figure 6).

Studies analyzing the effect of exercise upon adult neurogenesis have consistently demonstrated its positive effects in animals developmentally exposed to alcohol; in Boehme's study (2011), which used a rat model of alcohol exposure (4.3g/kg administered for the first and second trimesters, and 4.0g/kg for the third trimester) throughout all three trimesters of fetal development (as opposed to the third trimester-equivalent only model used in the current study), 12 days of wheel running enhanced dentate gyrus cell survival in control animals and alcohol-exposed animals (when analyzed at PD60). The rodent model of Boehme and colleagues (2011) exposes rats to alcohol for a longer period of development than the model featured in this study, and allowed animals to exercise for less time, but the authors were still able to see a robust increase in levels of neurogenesis. Additionally, in a rat model of first and second trimester-equivalent exposure, new cell proliferation and survival within the adult dentate gyrus were diminished, but both proliferation and survival were

increased in AE animals following exposure to wheel running (Redila et al., 2006). This improvement caused by exercise is mirrored when rodent behavior is analyzed; performance of rats in the Morris Water Maze was significantly improved, even in rats that had been exposed to alcohol during the third trimester-equivalent (Thomas et al., 2008). Additionally, Thomas and colleagues found that alcohol-exposed rats displayed overactivity when analyzed within an open field, suggesting increased anxiety in these animals, but that exercise had an anxiolytic effect on behavior.

A potential pathway through which exercise may induce its beneficial effects within the brain is through alterations of levels of brain-derived neurotrophic factor (BDNF). BDNF promotes the differentiation, neurite extension, and survival of a variety of neuronal populations, including that of the hippocampus (Barde, 1994). BDNF levels within the brain are altered depending upon a variety of factors, including alcohol exposure (Heaton et al., 2000; Feng et al., 2005; Caldwell et al., 2008). Also, exercise has been shown to increase levels of BDNF within the hippocampus and improve hippocampal function in rodent models and in humans (Coltman et al., 2002; Olif et al., 1998; Griffin et al., 2011). It is likely that increased levels of BDNF within the hippocampus are caused by exercise and are contributing to some of the positive effects of exercise seen in this model of voluntary exercise. Work from our own lab has demonstrated that increases in BDNF are found following the WR/WR intervention but not seen within WR/EC rats, supporting the idea that continuous aerobic exercise most affectively increases BDNF levels (Boschen et al., *unpublished data).* Additionally, alterations in the levels of other neurotrophic factors,

such as vascular endothelial growth factor (VEGF), has been found to stimulate neurogenesis (Jin et al., 2002). VEGF could be particularly relevant to this time point of neurogenesis, as a receptor for VEGF, Flk-1, is expressed on DCX+ cells (Jin et al., 2002), which suggests that VEGF is not only involved with increased microvasculature, it might affect DCX+ cells directly.

Despite these many examples of the hugely beneficial effects of exercise upon adult neurogenesis within the dentate gyrus, gaps in the literature remain. Notably, most studies use markers other than DCX to assess the impact of exercise following third trimester-equivalent alcohol exposure on adult neurogenesis (including Ki-67 and bromodeoxyuridine). Thus, it is possible that WR is a sufficient intervention for improving cell proliferations and survival in control animals, but has a more variable impact on the number immature neurons following our model of FASD.

4.3 Dendritic Complexity

Increases in total dendritic material in immature neurons that express DCX, as measured by changes in optical density of hippocampal sections stained immunohistochemically, were observed following the WR/WR intervention in the SC and SI groups, mirroring the observed increases in cell number. Interestingly, the WR/WR intervention did not increase levels of total dendritic material in the AE group. Total dendritic material was used as an indirect measure of dendritic

complexity in this study. Contrary to our hypothesis and previous work in our lab, neonatal alcohol exposure did not affect basal amounts of dendritic material.

Voluntary exercise and environmental complexity have been shown to induce positive changes in measures of dendritic complexity. A potential relationship between these interventions and their effect upon neurogenesis and complexity was explained by Olson and colleagues (2006); voluntary exercise affects dendritic spine density, alters the expression of synaptic proteins and neurotrophins, induces vascular changes and expression of VEGF, and alters learning and memory. These changes subsequently increase cell proliferation and neurogenesis. Additionally, environmental complexity affects dendritic spine density, expression of neurotrophins and synaptic proteins, and learning and memory, which, in turn, increase neurogenesis.

Beyond this hypothesis, experimental evidence has shown that voluntary exercise and environmental complexity have both been shown to increase dendritic complexity within rodent models. A study conducted by Eadie and colleagues (2005) found that the structural integrity and levels of dendritic complexity, as measured by spine density and dendrite length, were increased in adult rats when they were allowed free access to running wheels for two weeks as adults. These results were confirmed by the same lab (Redila and Christie, 2006), who found that dendritic complexity as measured by Sholl analysis and dendritic length increased in response to wheel running.

Previous work within our lab, which utilized the same alcohol exposure model as seen in this study, analyzed the same cell marker (DCX), and examined brains at

PD42 or PD72 following housing in behavioral interventions, produced different results. At PD42 and 72, it was found that AE decreased baseline dendritic complexity within the infrapyramidal blade of the dorsal dentate gyrus in DCX+ cells in SH animals (Hamilton & Klintsova, *unpublished data*). When rats were housed in WR from PD30-42, exercise diminished deficits seen in dendritic length and complexity. Housing in WR/EC (same timeline as used the current study), also increased dendritic length and complexity in AE animals back to control levels. Work in our lab investigating dendritic complexity in the medial prefrontal cortex also found that AE decreased baseline dendritic complexity (Hamilton et al., 2009).

Measures of total dendritic material were taken from dorsal hippocampus only in this study. Dorsal and ventral hippocampus exhibit differences in functionality based on regional differences in outputs and inputs. The dorsal hippocampus has been shown to relate primarily to cognitive functions and memory, while the ventral hippocampus regulates emotional and affective processes (Fanselow and Dong, 2010). We chose to focus on the dorsal hippocampus for this experiment as our lab has previously found that alcohol exposure reduced the number of adult-born neurons within the dorsal dentate gyrus (Klintsova et al., 2007). Additionally, the dorsal dentate gyrus has been shown to have higher levels of adult neurogenesis than the ventral dentate gyrus, and, the infrapyramidal blade of the DG has been shown to have higher levels of adult neurogenesis as compared to the suprapyramidal blade (Snyder et al., 2009). It has been suggested that these regional differences in amount of adult neurogenesis are indicative of differences in susceptibility to alcohol exposure, and the

infrapyramdial blade of the DG may be specifically targeted when rats are exposed to alcohol (Hamilton & Klintsova, *unpublished data*). Regional differences in vulnerability could explain why AE animals did not have decreased baseline dendritic complexity in the current study, as the brains were sectioned horizontally not allowing for assessment of differences between dentate gyrus blades.

Although there was no effect of alcohol exposure upon baseline levels of dendritic complexity within this study, we did find that within the AE/WR/WR group, dendritic complexity was not increased as compared to the AE/SH group, although an increase was seen in the SC/WR/WR group as compared to the SC/SH group and in the SI/WR/WR group as compared to the SI/SH group. This results highlights the deleterious nature of AE upon dendritic complexity; although a baseline decrease was not seen, alcohol exposure was damaging enough to prevent WR, which has been demonstrated many times to be an immensely positive intervention, from having an effect upon dendritic complexity.

The finding that WR/WR did not increase total dendritic material of DCX+ cells in the AE group is important due to the time point of neurogenesis being analyzed in this study. DCX is expressed in immature neurons, during the early part of a stage of neurogenesis that has been deemed "competitive survival" (Tashiro et al., 2006). During this time period, cells are competing for resources (such as oxygen, nutrients, and tonic GABAergic input) while extending neurites toward the molecular layer, which will eventually make synaptic connections onto axons entering from entorhinal cortex. An increased number of DCX+ cells indicates an increase in

immature neurons competing within this critical time period. An increase in the number of DCX+ cells is indicative of a better environment in which cells can mature; WR/WR may be increasing microvasculature and levels of VEGF and BDNF within the dentate gyrus, allowing for the observed increases in DCX+ cell number in the control group. In the healthy brain, exercise enhances functional connections within the hippocampal network, which may contribute to enhanced survival of cells during later stages of maturation and integration into the hippocampal network. The lack of observed increases in dendritic material within the AE-WR/WR group could suggest a deficiency in the ability of these neurons to benefit fully from the enhanced cellular environment. These deficits could manifest in decreased long-term cell survival caused by alcohol, which have been previously reported by our lab using BrdU labeling (30 days post-injection, Klintsova et al., 2007). When pairing the data collected in the current study with previously published reports from our lab regarding long-term cell survival, it suggests that alcohol exposure could be altering the cellular environment, interfering with the ability of the immature neurons to make functional connections, and thus inhibiting cell survival within the dentate gyrus.

4.4 Relevance to FASD in Humans

Fully understanding the brain's response to alcohol exposure, voluntary exercise, and environmental complexity in rodent models will give insight into how the process of adult neurogenesis in the human brain might respond to similar

environmental influences. Though the results observed in this study cannot be expected to model the phenomena of the human brain exactly, they do provide valuable insight. Wheel running, or voluntary exercise, has consistently been shown to be a valuable intervention in terms of brain plasticity, suggesting that regimens of exercise would be very beneficial towards humans with FASDs. It has been shown previously that a 5-week-long voluntary exercise regimen has increased the serum concentration of BDNF in young adult males (Griffin et al., 2011). Additionally, exercise has been shown to increase executive function, visuospatial memory, and pattern separation, and hippocampal blood volume within adult humans (for review, see Voss et al., 2013). It is likely that interventions of exercise positively increase adult neurogenesis within humans, as is seen in rat models, which results in the observed improvements in human cognition that are seen as a result of exercise. These data, combined with data from the rodent literature, suggest that exercise could be a viable behavioral intervention for behavioral and cognitive deficits seen in humans with FASD.

4.5 Conclusions and Future Directions

The goal of the current study was to obtain information on the effect of neonatal alcohol exposure, voluntary exercise, and environmental complexity upon measures of adult neurogenesis and dendritic material in the dentate gyrus of the hippocampus of the adult rat brain. It was expected that interventions of wheel running

and wheel running followed by environmental complexity would increase the number of DCX+ cells in suckle control, sham-intubated, and alcohol-exposed animals. Additionally, it was expected that alcohol would decrease the total amount of dendritic material, but that interventions of wheel running and wheel running followed by environmental complexity would increase the total amount of dendritic material in all animals, regardless of group. Contrary to our hypotheses, we observed that only wheel running increased the number of DCX+ cells in suckle control and sham-intubated animals. Additionally, wheel running increased the total dendritic material for SC and SI animals, but was not able to cause an increase within AE animals. These results indicate that alcohol exposure is deleterious enough to cause persistent impairment of brain plasticity into adulthood within the adult rat brain.

Although there has been much research concerning how alcohol exposure, wheel running, and environmental complexity influence adult hippocampal neurogenesis, we still do not know why exactly the dentate gyrus responds the way it does to environmental insults and stimuli. The *Bdnf* gene has been of particular interest to our lab, and has been implicated in the manifestation of the effects of alcohol, wheel running, and environmental complexity, as alcohol exposure may continually influence the synthesis and release of BDNF throughout the life of an individual (Goodlett et al., 2005). Additionally, as this study contained only male animals, it would be interesting to see if measures of adult neurogenesis and total dendritic material were altered in similar ways within the female brain. Long-term survival could also be assessed within this model using BrdU, to see how alcohol

exposure and environmental interventions impact long-term survival of newly-generated neurons. These measures and others can lead to a better understanding of the influence of developmental alcohol exposure upon the brain, and lead towards interventions that can positively influence the lives of those with FASDs.

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